FREE THYROID HORMONE AND SENSITIVE THYROTROPHIN MEASUREMENTS IN THE ASSESSMENT OF THYROID STATUS

by

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DECLARATION OF ORIGINALITY

This thesis was composed by myself and the work presented in it is my own. Measurements made by others as part of collaborative studies are as indicated in the text.

Sadie M Gow

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I would like to dedicate this thesis to the memory of Mrs Dorothy M Gray.

ABSTRACT

Developments in immunoassay have led to the availability of (a) analogue radioimmunoassays (RIA) which purport to measure the minute concentrations of free thyroid hormones in plasma and (b) highly sensitive immunometric assays for thyrotrophin (TSH) which could potentially distinguish, in a basal plasma sample, the normal TSH levels in euthyroidism from the suppressed levels found in hyperthyroidism. In this thesis, the performance of these tests has been assessed in terms of their analytical precision, accuracy and diagnostic effectiveness in various clinical settings and compared with standard tests of thyroid function. The results of these studies are summarised below:-

Free thyroid hormones

Measurements by several analogue RIAs were precise, robust and simple but significant differences in analytical accuracy were shown compared to direct equilibrium dialysis reference methods which were developed specifically for this evaluation. In uncomplicated patients presenting to an endocrine clinic, the free hormone results had a higher predictive value than those for total hormones. This was probably attributable to the free hormone measurements being unaffected by changes in plasma thyroxine-binding globulin. However, in pregnancy and in patients with systemic illness, many low values

were found by analogue RIA compared to equilibrium dialysis. The analogue assays were also affected by changes in albumin concentration and affinity, and also the presence of autoantibodies to thyroid hormones.

Sensitive TSH measurements

Two immunoradiometric assays (IRMA) for TSH were evaluated and these showed good reproducibility and analytical recovery with greater sensitivity, specificity and working range than RIA methods. Samples from hypothyroid patients with very high TSH could therefore be analysed without sample pre-dilution. Of more importance was the demonstration that basal TSH results by IRMA reliably predicted results of the TSH-releasing hormone test (TRH test) in hospital in-patients and patients attending a thyroid clinic for initial assessment or follow-up. They were also independent of changes in plasma proteins. A small number of undetectable TSH results was found in early pregnancy, the severely ill and in the elderly. The test was inappropriate in the early follow-up of patients treated for hyperthyroidism but in hypothyroid patients treated with thyroxine, it provided the best biochemical indicator of both over- and under-replacement.

Although measurement of free thyroid hormones represents a significant advance compared to total hormones, the measurement of basal TSH by immunometric assay provides a more sensitive and specific test in the diagnosis of thyroid dysfunction and in monitoring thyroxine

therapy. Advantages of (1) decreasing the number of unnecessary tests and (2) identifying subclinical disease has led to the proposal of a new strategy of thyroid function testing with basal TSH as the first-line test.

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Chapter 1

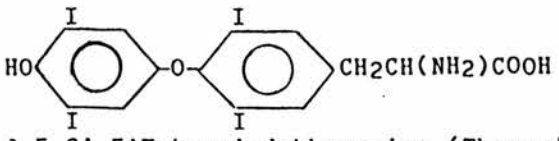
INTRODUCTION

1.1 THE HYPOTHALAMIC-PITUITARY-THYROID AXIS

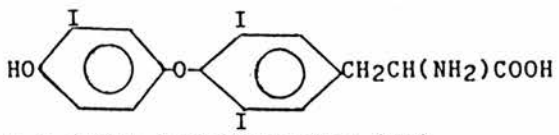
1.1.1 Thyroid Hormone Production and Metabolism

In man, the thyroid gland is the site of synthesis of all the circulating thyroxine (T_4) and 15% of triiodothyronine (T_3), most of the T_3 being derived from outer-ring monodeiodination of T_4 in the liver, kidney and muscle, Figure 1.1 (Sterling et al., 1970; Schimmel & Utiger, 1977).

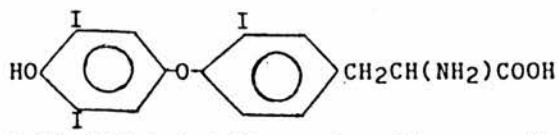
Thyroxine was thought originally to be the most important thyroid hormone, since its concentration in plasma is approximately 55-fold higher than T_3, and it has a longer half-life (7 days cf. 1 day). However, it has since been shown that T_3 is metabolically more active than T_4, binding to nuclear receptors with ten-fold greater affinity (Oppenheimer et al., 1976). Thyroxine is, therefore, regarded by many as a pro-hormone. Monodeiodination also occurs on the inner ring of T_4 giving the metabolically inactive isomer reverse T_3 (rT_3), 90% of which is formed in peripheral tissues (Schimmel & Utiger, 1977). The structures of T_4, T_3 and rT_3 are shown below:-



3,5,3',5'Tetra-iodothyronine (Thyroxine, T_4)



3,5,3'Tri-iodothyronine (T_3)



3,3',5'Tri-iodothyronine (Reverse T_3, rT_3)

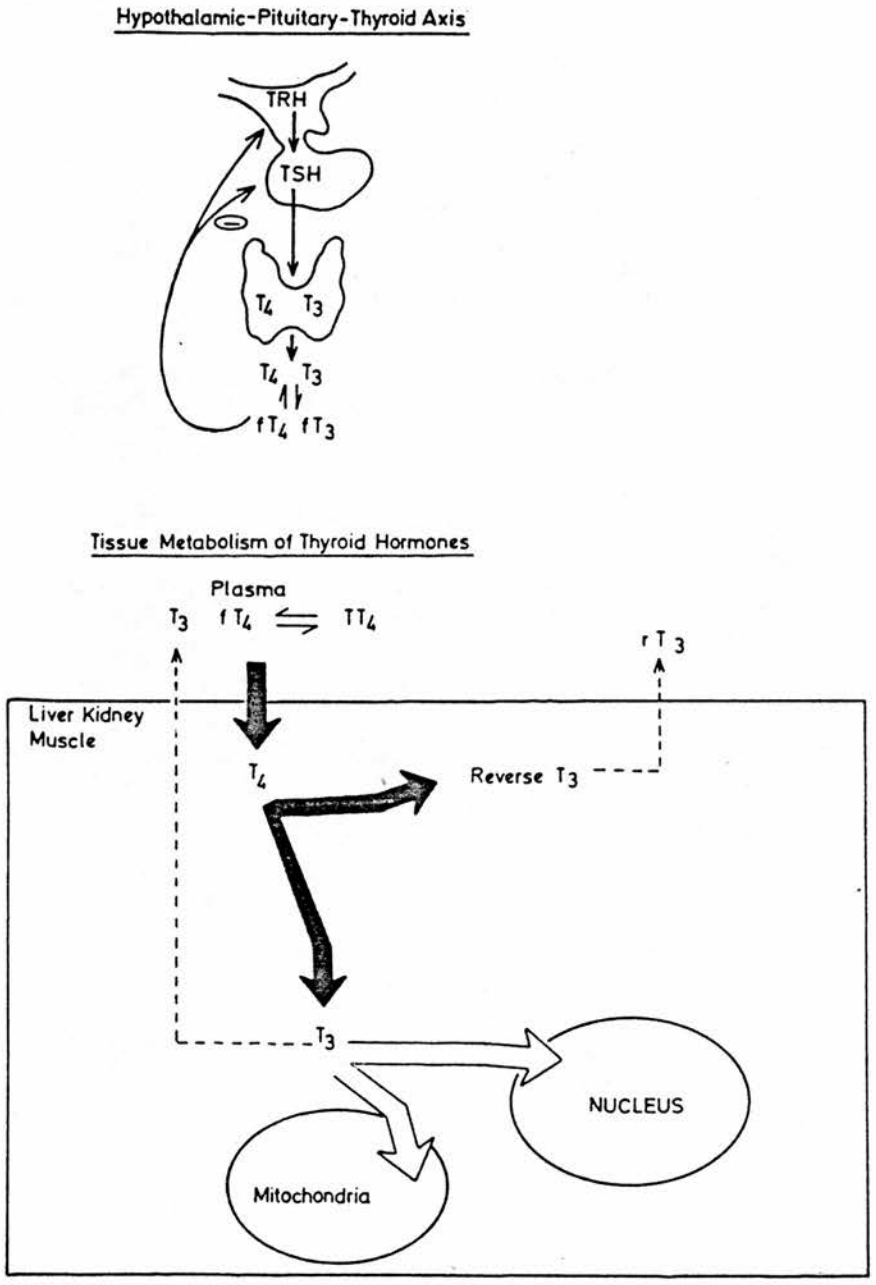


Figure 1.1 The Hypothalamic-pituitary-thyroid Axis and Tissue Metabolism of Thyroid Hormones.

In the cells of the thyroid gland, tyrosyl residues, which are attached to the large glycoprotein thyroglobulin (MW 670,000) in the colloid matrix, are iodinated and coupled together to form thyroid hormone precursors. The processes of iodine-trapping and oxidation, iodothyronine synthesis, colloid endocytosis, proteolysis and hormone secretion are all stimulated by thyrotrophin (TSH) secreted from the anterior pituitary gland (Figure 1.1). Thyrotrophin regulates adenyl cyclase activity by binding to high affinity receptors on the thyroid cell membrane. This increases the intracellular concentration of cyclic AMP which mediates the effects of TSH on thyroid hormone production (Lefort et al., 1984).

Thyroid hormones are excreted either unchanged by the kidney, as glucuronide and sulphate esters in bile or they are deiodinated and deaminated in the liver and kidney with re-cycling of the released iodide.

1.1.2 Thyroid Hormone Transport in Blood

Thyroid hormones are predominantly protein-bound in plasma. This protein-binding possibly facilitates transport to target tissues, prevents excessive loss into the urine or flooding into cells and may provide a readily available reservoir of hormone if needed (Hoffenberg & Ramsden, 1983). The specific α_2 globulin, thyroxine binding globulin (TBG), binds 70% and 80% of the plasma total T_4 and T_3, respectively (Table 1.1) with less

Table 1.1 The Characteristics of Thyroid Hormone-Binding Proteins in Plasma

	Binding Protein		
	TBG	TBPA	Albumin
MW (daltons)	54-65,000	50,000	66,000
Subunit structure	Monomer	Tetramer	Monomer
Binding Sites	1	2	1 High affinity 6 Low affinity
Plasma concentration	3.6×10^{-7} mol/l	4.6×10^{-6} mol/l	6.4×10^{-4} mol/l
Binding constant, K_a (l/mol)	T_4 6.0×10^9 T_3 3.3×10^8	T_4 7.0×10^7 (Site 1) T_4 6.7×10^5 (Site 2) T_3 1.4×10^7 (Site 1) T_3 5.5×10^5 (Site 2)	T_4 7.0×10^5 (Site 1) T_4 4.8×10^4 (Site 2-6) T_3 1.0×10^5 (Site 1) T_3 6.9×10^3 (Site 2-6)
Distribution of binding - T_4 - T_3	70% 80%	20% 10%	10% 10%

Data from Hoffenberg & Ramsden, 1983 and Kamikubo et al., 1984.

binding to thyroxine-binding prealbumin (TBPA) and the low affinity, high capacity sites of albumin. The high affinity and capacity of the binding proteins results in less than 0.05% T_4 and 0.5% T_3 circulating in the free form, but it is generally accepted that it is this fraction which can cross plasma membranes and affect intracellular metabolism (Robbins & Rall, 1979). Theories of the kinetics of this process are still controversial, the relative importance of the dissociation of T_4 from albumin, TBPA and TBG in the capillaries being disputed (Ekins et al., 1982; Pardridge, 1982).

In pregnancy, the TBG concentration and hence the total thyroid hormone concentrations in blood rise whereas the free levels remain relatively constant (Ekins et al., 1982). This has led to further speculation as to the physiological role of TBG and whether a rise in hormone-binding proteins redirects hormone delivery to either tissues with long capillary transit times (e.g. the liver) to meet the requirement for increased protein synthesis in pregnancy (Pardridge, 1982) or to the foetus before the development of the foetal thyroid (Ekins et al., 1982).

Although entry of thyroid hormones into cells has previously been regarded as a passive process, active transport of thyroid hormones via membrane receptors has been reported in rat hepatocytes (Rao et al.,1976; Krenning et al., 1981) and cultured fibroblasts (Cheng et al., 1980).

The concept of the free hormone concentration being the sole determinant of cellular utilisation, therefore, represents an over-simplification but provides a working model to explain the clinical findings observed in situations where changes in protein binding cause large changes in the total but not the free hormone concentrations, for example in pregnancy, and in the benign familial conditions of TBG excess and deficiency (Burr et al., 1980). The overall metabolic status in such situations correlates more closely with the plasma free hormone fraction than with bound hormone. The availability of routine tests of the free hormone level has therefore been regarded as most important by clinicians and clinical chemists.

1.1.3 The Cellular Action of Thyroid Hormone

Thyroid hormones exert a wide range of physiological effects for example stimulating somatic growth, neural development, oxygen consumption, heat production and regulating cellular metabolism. Their mode of action at the cellular level remains unclear although binding sites have been demonstrated in the cytosol (Hamada et al., 1970), nucleus (Oppenheimer et al., 1972), mitochondria (Sterling & Milch, 1975), plasma membrane (Pliam & Goldfine, 1977) and endoplasmic reticulum of rat liver cells (Fekkes et al., 1979).

The binding of T_3 to nuclear receptors in the chromatin increases mRNA transcription (Tata & Widnell,

1966; Dillman et al.,1978) and subsequent increases have been shown in the activities of malic enzyme in the cytosol and glycerophosphate dehydrogenase and cytochromes in the mitochondria of rat liver (Degroot, 1979). The strongest evidence in favour of the central role of T_3 nuclear receptors in hormone action, is that children with peripheral resistance to thyroid hormones have absent or abnormal receptors in their lymphocytes (Degroot, 1979).

When compared to nuclear receptors, the cytosolic binding proteins for thyroid hormones have low affinity but a high capacity (Hamada et al., 1970). Initially, it was thought that these were not intracellular transport proteins analogous to those described for steroid hormones (O'Malley et al., 1972) but acted solely as a reservoir (Dillman et al., 1974; Degroot, 1979). However, more recently, evidence for a role in transport between the cytosol and mitochondria has been reported (Hashizume et al., 1986).

Whether T_3 has direct effects on ATP generation and oxidative phosphorylation via the binding sites in the mitochondria is still a matter of controversy (Degroot, 1979; Menezes-Ferreira & Torresani, 1983; Hashizume et al., 1986). The plasma membrane Na^+/K^+-ATPase is activated by thyroid hormone and it is argued that depletion of the ATP pool stimulates mitochondrial respiration, oxygen consumption and thermogenesis (Smith & Edelman, 1979).

1.1.4 Regulation of Thyroid Hormone Secretion

Thyroid gland activity is controlled by the plasma TSH level which, in turn, is regulated by thyrotrophin-releasing hormone (TRH) and the negative feedback control of free thyroid hormones (Figure 1.1).

Thyrotrophin is a glycoprotein (MW 28,000) composed of two dissimilar polypeptide subunits (α and β) and 20% carbohydrate. The α subunit is immunologically similar and structurally homologous with the α subunit of human chorionic gonadotrophin (HCG), luteinizing hormone (LH) and follicle stimulating hormone (FSH); the β subunit possesses the biological activity of TSH but requires combination with the α subunit for activity (Pierce & Parsons, 1981). The secretion of TSH from the thyrotrophs is pulsatile as shown from recent work (Greenspan et al., 1986) and is modulated by TRH via the hypothalamic-pituitary portal system (Harris et al., 1978). The glycoprotein composition of TSH is also regulated by TRH and this may affect its hepatic clearance and therefore its biological activity (Gesundheit et al., 1986).

The tripeptide TRH (pyroglutamyl-histidyl-prolinamide) is derived from a larger pro-hormone which has recently been localised in higher brain centres and the olfactory lobes (Lechan et al., 1986). Release of TRH in the hypothalamus is thought to be regulated by

neural input (Morley, 1981), and possibly by thyroid hormones (Larsen, 1982). Its action at the thyrotroph may be further modulated, however, by thyroid hormones and the hypothalamic regulators, dopamine and somatostatin (Morley, 1981; Greenspan et al., 1986). Regulation of the activity of the hypothalamic-pituitary-thyroid (HPT) axis by changes in temperature, day length and nutritional status probably involves several of these mechanisms. Extracellular calcium is required for TRH to induce TSH release, involving the inositol phosphate-calcium signalling system (Berridge, 1984; Gershengorn & Paul, 1986).

The negative feedback control of thyroid hormones on the pituitary is the main regulator of TSH secretion. Administration of T_3 and T_4 results in a rapid decrease in TSH secretion and, over a prolonged time-period, TSH synthesis is suppressed due to inhibition of the synthesis of TSH messenger RNA (Larsen, 1982; Gurr et al., 1986). Mechanisms postulated for this inhibition are:- (1) the synthesis of an inhibitory protein which interferes with the cell's response to TRH (Vale et al., 1968; Reichlin, 1978) and (2) inhibition by T_3 of calcium uptake by the thyrotroph, leading to uncoupling of TRH from its second messenger system (Larsen, 1982).

In the liver and kidney, most of the nuclear T_3 is derived from plasma T_3 whereas in the pituitary, about

50% is derived from local T_3 production (Silva et al., 1978). In addition, there is greater nuclear receptor occupancy by T_3 in the pituitary (80%) than in the liver or kidney (50%). The pituitary gland may, therefore, be much more sensitive to circulating T_4 levels than other peripheral tissues (Larsen, 1982; Kaplan, 1984).

1.2 DISORDERS OF THYROID FUNCTION

1.2.1 Primary Thyroid Failure

The main causes of primary hypothyroidism are: spontaneous atrophy of the thyroid, Hashimoto's thyroiditis and following treatment of hyperthyroidism. All forms are about ten times more common in women and there is a higher incidence in the elderly (Tunbridge et al., 1977). Spontaneous atrophy of the thyroid is associated with other autoimmune disorders (pernicious anaemia, diabetes mellitus, Addison's disease). Lithium therapy used for depressive illness can also lead to hypothyroidism as it inhibits thyroid hormone synthesis.

In some clinically euthyroid patients, there is impaired thyroid reserve and TSH secretion is increased to maintain thyroid hormone concentrations. If not drug-induced, this "subclinical hypothyroidism" slowly progresses to overt hypothyroidism (Toft et al., 1981). Thyroxine replacement therapy is usually withheld in such patients until there is biochemical and clinical evidence

of hypothyroidism (e.g. weight gain, tiredness, cold intolerance, bradycardia etc.).

1.2.2 Secondary Hypothyroidism

In rare cases, hypothyroidism may be secondary to anterior pituitary failure (as a result of hypophysectomy or a pituitary adenoma) or hypothalamic disease. An expanding pituitary tumour tends to alter the secretion of other pituitary hormones before TSH, with resultant clinical manifestations.

1.2.3 Hyperthyroidism

Hyperthyroidism is commonly due to Graves' disease, toxic multinodular goitre and, less frequently, an autonomous solitary nodule. All forms occur about six times more often in women than men. Graves' hyperthyroidism, which tends to be more severe, presents at an earlier age (30-50 years). Immunoglobulins in the serum of patients with Graves' disease (a) bind and stimulate the TSH receptor, (b) stimulate growth of the thyroid and (c) bind and cause hypertrophy of eye muscle. Their varying titres cause most patients to have goitres and about half to have ophthalmopathy. Some euthyroid patients may exhibit only the eye signs of Graves' disease (Ophthalmic Graves' disease). The hyperthyroidism of toxic multinodular goitre develops insidiously from the proliferation of foci of thyroid follicles with excessive iodine turnover. The inhibition of TSH secretion due to a thyroid

adenoma results in atrophy of the rest of the thyroid. Depending on the aetiology, history and age of the patient, hyperthyroidism is treated with radioiodine, surgery or anti-thyroid drugs e.g. carbimazole which blocks the oxidation of iodide. Hyperthyroidism due to hypersecretion of TSH from the pituitary, is very rare.

Patients with suppressed TSH secretion but normal thyroid hormone levels are described as having subclinical hyperthyroidism, and those with multinodular goitre or a solitary nodule may progress to overt disease with symptoms of weight loss, heat intolerance, palpitation, tremor etc. Anti-thyroid treatment is considered in such patients if there is unexplained atrial fibrillation or an autonomous nodule (Toft et al., 1981).

1.3 BIOCHEMICAL TESTS OF THYROID FUNCTION

It can be seen from the physiology of the HPT axis, that the level of thyroid activity can be assessed in two ways i.e. by measurement of circulating thyroid hormone concentrations or by determining the end-organ response; this is most readily achieved by measuring thyrotroph responsiveness. If thyroid dysfunction is detected, other tests may be required to indicate the cause of the disease e.g. thyroid isotope scans and measurement of serum autoantibodies. In the past few years, there have been considerable improvements in our

ability to measure thyroid hormone and TSH concentrations in plasma. These will now be reviewed.

1.3.1 Total Thyroid Hormone Measurements

Prior to the availability of specific anti-T_4 antisera in the 1960s, most laboratories estimated the protein-bound iodide of plasma by releasing the iodide with acid and quantitating its catalytic effect on the cerate-arsenite reaction (Blackburn & Power, 1955) but this was a hazardous procedure which gave misleadingly high results in the presence of iodine-containing drugs or X-ray contrast media (Toft et al., 1973). Competitive protein binding assays for T_4 using TBG as the binding agent had the disadvantage of requiring extraction of serum before assay and problems of cross-reaction with T_3 were encountered. The technique of radioimmunoassay (RIA), first described by Yalow and Berson in 1960, permitted the specific measurement of both total T_4 and T_3 in unextracted plasma.

In general, total T_4 measurements discriminated both hyper- and hypothyroidism adequately from the euthyroid state, with total T_3 being a particularly valuable investigation in suspected hyperthyroidism since twice the increase in values, above the upper limit of normal, were found with T_3 compared to T_4 (Toft et al., 1973; Seth et al., 1976). In hyperthyroidism, thyroidal T_3 production is increased, as is the extra-thyroidal pool

size of T_3, which may account for the greater rise in plasma levels. Total T_3 measurements have also proved useful in the relatively rare cases of T_3-thyrotoxicosis, where total T_4 concentrations are normal. The measurement of T_3 is of little value in investigating hypothyroidism where normal levels are often found (Seth et al., 1976).

Since total thyroid hormone levels change with alterations in protein binding, these measurements give misleadingly high results due to oestrogen-induced increases in TBG e.g., in patients who are pregnant or receiving oral oestrogens (e.g. the oral contraceptive pill) or who have the rare condition of hereditary TBG excess. Euthyroid hyperthyroxinaemia due to abnormally increased binding to TBPA (Moses et al., 1982) and albumin (Stockigt et al., 1981) has also been described recently.

Misleadingly low total thyroid hormone concentrations occur in patients with hereditary TBG deficiency and in hypoproteinaemic states such as chronic liver disease and nephrotic syndrome. Some drugs cause low values by displacing T_4 and T_3 from their binding sites (e.g. salicylate, fenclofenac), while others e.g. androgens, corticosteroids and phenytoin increase the clearance of thyroid hormones from plasma. Levels of total T_3 are also frequently reduced in non-thyroidal illness due to decreased peripheral conversion of T_4 to

T_3 (Chopra et al., 1975). This has confused the issue as to whether there is an age-related decline in plasma total T_3 concentrations (Olsen et al., 1978). Fasting also reduces total T_3 levels due to decreased 5'-deiodinase activity (Braverman & Vagenakis, 1979).

In order to overcome the problems with total thyroid hormone measurements due to changes in protein binding, several indirect and direct methods for estimating the free fraction have been devised.

1.3.2 Free Thyroid Hormone Measurement

(a) Indirect Methods

Indirect methods are those in which the free hormone concentration is calculated from measurements of both the total hormone concentration and either the percentage free fraction (commonly measured by equilibrium dialysis, ultrafiltration or sephadex adsorption) or the total serum binding capacity. A classification of indirect methods according to Ekins (1979) is shown in Table 1.2a. Although methods for quantitating the free fraction were developed in the 1960s (Sterling & Hegedus, 1962), these were too technically demanding for routine applications (see 1.3.2b). Consequently, measurements of either the serum binding capacity by an uptake test or the TBG concentration, with calculation of the free T_4 index (FT_4I) or T_4:TBG ratio, were widely adopted.

Table 1.2 Free Thyroid Hormone Measurements

(a) INDIRECT METHODS

Equilibrium:

1. Measurement of total T_4 or T_3 and the percentage free hormone using radiolabelled hormone as indicator after dialysis, ultrafiltration etc.

2. Measurement of total T_4 and serum binding proteins to give either T_4:TBG ratio or a calculated free hormone concentration from known binding constants.

3. Measurement of total T_4 or T_3 and the unoccupied binding sites to give FT_4I or FT_3I.

Dynamic:

1. Measurement of the rate of transfer of radiolabelled hormone from one compartment to another (Ross, 1978).

2. Measurement of the rate of association of labelled hormone to antibody in the presence and absence of a binding protein blocking agent (e.g. Corning Immophase Rate Method).

(b) DIRECT METHODS

Equilibrium:

1. Quantitation (by RIA or gas chromatography) of free hormone after separation from the bound hormone by dialysis, ultrafiltration or sephadex adsorption (e.g. Lepetit Lisophase sephadex column method).

Dynamic:

1. Sequential exposure of antiserum first to serum and then to back titration with label. Separation of antiserum from serum is either by a dialysis membrane or by using a solid-phase antibody (e.g. Clinical Assays two-step method).

Equilibrium Immunoassay:

2. Simultaneous incubation of antiserum, label and sample with separation of antiserum from serum binding proteins effected by:-
   - microencapsulation of antibody (e.g. Damon Liquisol method)
   - use of a chemically-modified T_4 radiolabel which does not bind to serum binding proteins (e.g. Amerlex analogue method)

Thyroid hormone uptake tests involved determining the partition of radiolabelled T_3 between plasma proteins and an adsorbent to estimate the unoccupied binding sites (U-TBP). The equilibrium between free T_4, T_4 bound to protein (TBP-T_4) and U-TBP can be described as:

$$\text{Free } T_4 + \text{U-TBP} \rightleftharpoons \text{TBP-}T_4$$

Since the equilibrium favours protein binding, the total T_4 by RIA effectively measures [TBP-T_4]. From the law of mass action, therefore:-

$$[\text{Free } T_4] = \frac{[\text{Total } T_4]}{K\,[\text{U-TBP}]}$$ where K is the association constant, and the $FT_4I = \frac{[\text{Total } T_4]}{[\text{U-TBP}]}$

The FT_4I has been shown to correct for small changes in TBG levels e.g. due to the effects of oestrogens but not for the changes in protein binding found in sick, elderly patients (Witherspoon et al., 1981) or those with hereditary TBG abnormalities (Burr et al., 1977). Although the T_4:TBG ratio is satisfactory for wide changes in TBG concentration, two measurements still have to be made with combination of the results. These tests have therefore not replaced total T_4 measurements but are used in certain patients if a TBG abnormality is suspected.

Other indirect methods (Table 1.2a), involved measurements of the rate of dialysis of radiolabelled hormone from serum to a buffer compartment or the rate of association of radiolabel to an added antibody. Although

shorter incubations compared to equilibrium dialysis were possible with the dialysis-rate method of Ross (1978), it has not been widely used. Corning Medical developed the first free T_4 (fT_4) kit in 1978 which involved the measurement of the rate of association of labelled T_4 to antibody coupled to glass beads in the presence and absence of a serum binding protein blocking agent. The former represents a total T_4 measurement, and the latter was assumed to be proportional to the fT_4 concentration, the ratio being compared to that for standards calibrated against equilibrium dialysis. This kit, however, lost favour in the light of the low values found in pregnancy sera (Boss & Kingstone, 1979), abnormal values found in patients with congenital TBG abnormalities (Witherspoon *et al.*, 1980; Symons *et al.*, 1983) and criticisms of the mathematical manipulations employed to calculate results.

Until the availability of high avidity antisera in the late 1970s, the indirect equilibrium techniques, which do not rely on calibration (Table 1.2a), were regarded as the reference methods for free thyroid hormone measurement.

(b) Sources of Methodological Error in Early Reference Methods

There is considerable variation in reported normal values for fT_4 when radiolabelled (^{131}I or ^{125}I)

hormone is used to measure the free hormone fraction (Table 1.3a). This can be attributed partly to the presence of radioactive contaminants (mainly iodide and iodothyronine breakdown products) which do not reflect the distribution of unlabelled hormone between bound and free compartments in the dialysis or ultrafiltration system. Steps taken to reduce this interference were (a) chromatography of the dialysates prior to radioassay (Sterling & Hegedus, 1962), (b) the use of diluted serum to dilute out the effects of tracer impurities (Oppenheimer & Surks, 1964) and (c) concentration of the T_4 in dialysates either by use of the thyroxine-binding capacity of serum (Ingbar et al., 1965) or precipitation of iodothyronines with magnesium chloride (Sterling & Brenner, 1966). Purification of the radiolabel by prior dialysis or specific immunoprecipitation, and immunoprecipitation of the serum dialysate to remove contaminants, further reduced the estimates of fT_4 by indirect dialysis procedures to 0.03% (Schussler & Plager, 1967; Beckett et al., 1983a).

Fewer indirect assays for free T_3 (fT_3) have been developed and the lack of consensus in the accepted reference range for total T_3 in serum has contributed to the greater divergence in fT_3 values reported by early workers (Table 1.3b).

Disagreement about the effects of serum dilution on the measured percentage of fT_4, highlighted further

Table 1.3 Indirect Methods for Free Thyroid Hormones and the Normal Concentrations Measured

(a) Free T_4

Reference	Separation Method (Overall Dilution of Serum)	Purification Technique	Normal Free T_4 (pmol/l) Mean±SD
Sterling & Hegedus, 1962	Dialysis (1/2)	Resin, paper chromatography	129±39
Oppenheimer & Surks, 1964	Dialysis (1/150)	TCA/serum precipitation	30
Ingbar et al., 1965	Dialysis (1/2)	Resin dialysis/serum precipitation	52±14
Sterling & Brenner, 1966	Dialysis (1/3)	$MgCl_2$ precipitation	54±10
Schussler & Plager, 1967	Ultrafiltration (neat)	TCA/serum precipitation	27± 5
Thorson et al.,1972	Ultrafiltration (neat)	$MgCl_2$ precipitation	55±10
Irvine, 1974	Sephadex (1/100)	-	35± 8
Pedersen, 1974a	Ultrafiltration (2/5)	Resin dialysis/serum precipitation	35± 6*
Sophianopoulos et al.,1980	Ultrafiltration (1/20)	Haemoglobin-charcoal adsorption	19± 6
Beckett et al.,1983a	Dialysis (1/3)	Immunoprecipitation	29±11
Wang et al.,1985	Ultrafiltraton (1/10)	Sephadex gel filtration	25± 8

(b) Free T_3

Reference	Separation Method (Overall Dilution of Serum)	Purification Technique	Normal Free T_3 (pmol/l) Mean±SD
Ingbar et al.,1965	Dialysis (1/2)	Resin, paper chromatography	0.9*
Nauman et al.,1967	Dialysis (1/150)	$MgCl_2$ precipitation	23±6.1
Oddie et al.,1971	Dialysis (1/3)	$MgCl_2$ precipitation	4.6±0.2
Pedersen, 1974a	Ultrafiltration (2/5)	Resin dialysis/serum precipitation	7.7±1.3*
Sophianopoulos et al.,1980	Ultrafiltration (1/20)	Haemoglobin-charcoal adsorption	2.9±0.8
Beckett et al.,1983a	Dialysis (1/3)	Immunoprecipitation	7.3±2.4
Wang et al.,1985	Ultrafiltration (1/10)	Sephadex gel filtration	3.1±0.8

*Calculated value assuming mean total T_4 of 100 nmol/l and total T_3 of 1.8 nmol/l

factors influencing the T_4 binding equilibrium. Oppenheimer and Surks (1964) demonstrated mathematically that serum dilution should have a relatively minor effect on the fT_4 concentration, due to the very small percentage of total T_4 existing in the free state. Experimentally, however, lower values for fT_4 were found in dilute serum (Oppenheimer & Surks, 1964; Ingbar et al., 1965) and this was thought to be due to a reduction of contamination in the dialysate of non-T_4 radioactivity. Spaulding and Gregerman (1972) showed that the dialysable fraction increased with increasing concentrations of phosphate and particularly chloride ion and reasoned that, when phosphate buffer is used to dilute serum prior to dialysis, endogenous chloride ions are progressively diluted and replaced by buffer ions with a consequent decrease in the measured fT_4. The different ionic composition of the buffers used to dilute serum in the methods outlined in Table 1.3, therefore, represents another source of methodological variation in addition to the degrees of purification of the radiolabel (Pedersen, 1974b).

The pH and temperature sensitivity of the binding of T_4 to binding proteins shown in early studies (Schussler & Plager, 1967; Spaulding & Gregerman, 1972), led to more rigorous maintenance of incubations at 37°C and at pH 7.4. Temperature control was easier using equilibrium dialysis than ultrafiltration but it was

recognised that ultrafiltration had the advantage of shorter incubations (approx. 1 h) compared to dialysis (approx. 24 h) where the possibility of the degradation of labelled iodothyronines, denaturation of protein and bacterial contamination was increased. With both techniques, practical difficulties still remained in avoiding the leakage of protein-bound hormone through the dialysis membrane or ultrafilter and in reducing the absorption of hormone onto the ultrafiltration apparatus.

It is clear, therefore, that free thyroid hormone values obtained by any indirect method can only be regarded as numerical indices of the absolute concentrations in blood and will be defined by the set of analytical conditions used.

(c) The Validity of Direct Equilibrium Methods

The direct measurement of fT_4 and fT_3 following the physical separation at equilibrium of the free and bound moieties (by dialysis, ultrafiltration etc) has been facilitated by the development of highly sensitive RIA techniques (Table 1.2b). Modifications to the original method by Ellis and Ekins (1975) are shown in Table 1.4 together with the normal values obtained. In spite of the different diluents and separation methods used in the RIAs, these direct methods gave values which were in reasonably close agreement but generally lower than those obtained by indirect reference

Table 1.4 Direct Equilibrium Methods for Free Thyroid Hormones

Reference	Method	Overall Serum Dilution	Buffer	RIA Separation	Normal Values, Mean ± SD (pmol/l) fT_4	fT_3
Ellis & Ekins, 1975	Dialysis	1:26	0.01 M HEPES pH 7.4	Dextran-coated charcoal	15.8± 5.3	7.7± 1.1
Jiang & Tue, 1977	Dialysis	1:10	0.07 M Phosphate pH 7.4	Dextran-coated charcoal	9.8± 2.2	-
Yeo et al., 1977a	Dialysis	1:26	0.01 M Phosphate pH 7.4	Dextran-coated charcoal	10.3± 3.1	10.1± 2.8
Petersen et al., 1977	Dialysis	1:25	+Phosphate pH 7.4	*Gas chromatography	24.1	6.4
Weeke & Orskov, 1979	Dialysis	1:24	KRB pH 7.4	Wick chromatography	16.5± 4.4	6.8± 1.4
Romelli & Pennisi, 1979	Sephadex adsorption	1:2	0.1 M Phosphate pH 7.4	Dextran-coated charcoal	14.3± 2.4	6.5± 1.5
Obregon et al., 1981	Dialysis	1.26	0.01 M HEPES pH 7.4	Dextran-coated charcoal	15.3± 2.8	-

+No molarity stated

*After dialysis, the free hormones were measured by gas chromatography rather than RIA

methods (Table 1.3). This direct approach avoids problems due to contaminants of the radiolabel in the dialysis system and the error involved in combining two results i.e. total T_4 and the percentage free fraction.

All direct equilibrium methods for fT_4 measurement described to date use diluted serum. This dilution will produce a perturbation of the equilibrium originally present in undiluted serum. In a similar way, sequestration of T_4 by the addition of absorbents in chromatographic methods (e.g. Lepetit) or by addition of a specific antibody in the equilibrium immunoassay techniques (see later), will enhance this disturbance. However, mathematical modelling of the T_4 binding equilibrium between all the serum binding proteins (Geiseler & Ritter, 1983; Kamikubo et al., 1984), suggests an absence of any substantial dependence of fT_4 on dilutions up to 50-fold and this has been confirmed experimentally by the direct dialysis-RIA techniques of Ellis and Ekins (1975) and Weeke and Orskov (1979). Since T_3 is more weakly bound to protein than T_4 with 0.3-0.5% circulating in the free form, it is likely that serum dilution will cause a greater perturbation of the equilibrium. Indeed it has been shown both theoretically and experimentally, that a 50-fold dilution of serum results in a 20% decrease of fT_3 values (Ellis & Ekins, 1975; Weeke & Orskov, 1979; Kamikubo et al., 1984). Since alterations

in buffer ion concentrations with serum dilution affect the binding equilibria, there has been a move towards the use of 0.01 M HEPES buffer, pH 7.4 for direct dialysis methods.

A second consequence of sample dilution is simultaneous dilution of serum constituents which, by interacting with TBG and other binding proteins, modulate the fT_4 concentration in undiluted serum. This is of particular importance in patients taking certain drugs that are known to interact with T_4-binding proteins, and in non-thyroidal illness where inhibitors of binding may be present (Ekins, 1979; Chopra et al., 1982).

It is evident that results by any methodology may not represent the true free hormone concentration present in undisturbed, undiluted serum. It is argued, however, that the direct equilibrium dialysis and ultrafiltration techniques come closest to satisfying the physicochemical criteria of validity applicable to a free hormone assay method (Ekins, 1979 & 1983a) and, as such, have been widely accepted as the best reference methods for fT_4 and fT_3 measurement.

(d) Direct Dynamic Free T_4 Methods

As an alternative to using a dialysis membrane or ultrafilter to separate protein-bound from free hormone, the reaction between radiolabel and serum binding proteins is prevented in the commercial Clinical Assays two-step

fT_4 method, by a sequential exposure of assay antibody first to serum and subsequently to labelled material. The antiserum is adsorbed to the walls of plastic tubes and serum is readily removed from the antibody after the first incubation by aspiration and washing. The unoccupied antibody binding sites are then back-titrated with ^{125}I-T_4 in the second stage. This assay, which first became available in 1979, is unaffected by serum dilution but has not been used widely due to the vulnerability of the assay to drift, variations in the timing of incubations and the practical inconvenience of sequential incubations (Ekins, 1983a).

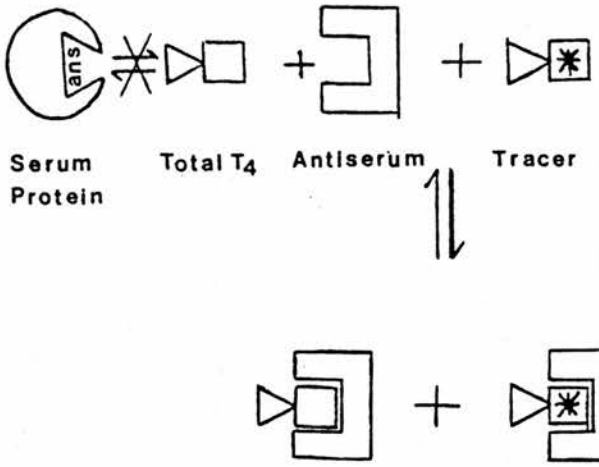
(e) Micro-encapsulated Antibody Methods

An alternative methodology marketed in 1979 by Damon Diagnostics utilises anti-T_4 antiserum micro-encapsulated within a semi-permeable membrane. Radiolabelled T_4 is bound to this antiserum and, in the presence of serum, the amount of ^{125}I-T_4 displaced from the antibody to the outside of the microcapsule, is proportional to the fT_4 concentration in the sample. Free and bound antigen can then be separated by centrifugation. This system has been likened to equilibrium dialysis in which the diffusion rate is 400 times faster (Ashkar et al., 1979) but assay results so far have not been completely independent of the concentration of total T_4 and of endogenous binding proteins (Witherspoon et al.,

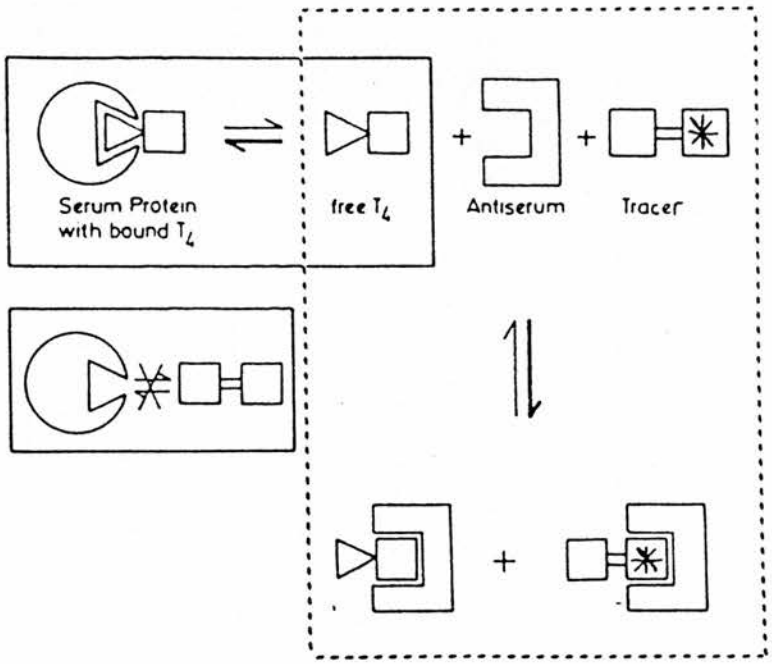
1980). Poor precision has also resulted from the fragility of the microcapsules.

(f) Equilibrium Analogue RIA Methods

Commercial assays which are being increasingly used are those which use simultaneous incubation of serum, and a labelled hormone analogue which binds to the assay antibody in the normal way but is chemically restricted from reaction with serum binding proteins (Midgley & Wilkins, 1981). The principle of this technique is contrasted with that of a conventional RIA in Figure 1.2. In a conventional RIA, a small mass of radiolabelled antigen which is similar or identical to the analyte is incubated with the analyte and a limited amount of specific antibody (Figure 1.2a). By the law of mass action, the amount of radiolabel bound to the antibody at equilibrium is inversely proportional to the analyte concentration in the sample (Ekins, 1974). Provided the antibody-bound ligand can be separated from the free ligand, the distribution of radiolabel can be quantitated and calibrated against standard analyte concentrations. In the RIA of total T_4, a protein-binding inhibitor e.g. anilinonaphthalene sulphonic acid (ANS) is used to dissociate T_4 from TBG and allow equilibration with the radiolabel. In the fT_4 analogue RIA (Figure 1.2b), the labelled T_4 derivative only equilibrates with the fT_4 pool and, therefore, the binding to the antibody is inversely proportional to the free hormone concentration.



(a) ANS displaces all of the T_4 bound to serum proteins which then equilibrates in the RIA.



(b) The equilibrium between serum binding-proteins and T_4 is not perturbed and the radiolabel cannot bind to serum protein.

Figure 1.2 The Principle of a Conventional Total T_4 RIA (a) and of a Labelled Analogue RIA for Free T_4 (b).

The amount of label bound to the antibody in (a) and (b) is inversely related to the concentration of the analyte.

In this analogue assay system, provided the antibody binding does not perturb the equilibrium significantly (i.e. <1% of total T_4 antibody-bound) and the serum proteins do not bind the analogue, this method should provide a valid fT_4 measurement (Ekins, 1983a). Amersham International produced the first analogue fT_4 assay and subsequently similar kits have been marketed by other manufacturers differing mainly in the hormone analogue, the reference method used for calibration of standards and the RIA separation system. Initial clinical studies suggest that these assays are independent of changes in TBG concentration (Wilke, 1982) and are technically convenient. They are consequently replacing the FT_4I in routine clinical laboratories and their wider application in a variety of clinical situations is now being actively researched. However, there is much controversy pertaining to the validity of such assays (Stockigt et al., 1982a & b; Amino et al., 1982; Midgley & Wilkins, 1982).

The development of commercial kits for fT_3 has been less prolific, the analogue methods being the main alternative at present (a column adsorption method by Lepetit has proved too slow and technically demanding for routine use). This has largely been due to the fact that the clinical utility of this test has yet to be fully established.

1.3.3 Basal Thyrotrophin Measurements

The release of TSH by the pituitary thyrotrophs is a sensitive indicator of the adequacy of circulating thyroid hormone levels. In the mid-1960s, purification of human TSH led to the generation of TSH antisera and the replacement of bioassays with specific RIA systems (Odell et al., 1965). These systems are sufficiently sensitive to detect elevated TSH concentrations but preparation of serum concentrates (Wehmann et al., 1979) or use of extended incubation times (Wide & Dahlberg, 1980) are needed to distinguish, reliably, suppressed values from normal; these do not provide convenient methods for routine investigations. The main clinical application of TSH measurements by RIA has therefore been in the detection of primary hypothyroidism.

Many antisera to TSH show some cross-reaction with other human glycoprotein hormones, particularly LH and HCG and this has also limited the attainable sensitivies of RIAs. This may be due to similar epitopes on the different hormones or to the preparation used as an immunogen being only partially pure. This problem has been circumvented by absorbing the TSH antiserum with an excess of HCG to saturate cross-reacting LH binding sites (Odell et al., 1965; Wehmann et al., 1979).

In patients with primary hypothyroidism, the TSH concentration in serum is raised regardless of the

aetiology (Hersham & Pittman, 1971). In mild or sub-clinical hypothyroidism, total T_4 concentrations are within normal limits, and the increase in TSH secretion is the first measurable sign of declining thyroid secretion (Irvine et al., 1973). Slight changes in thyroid hormone concentration within the population reference range may, therefore, produce marked effects on the negative feedback system and TSH secretion of the individual (Snyder & Utiger, 1972a; Saberi & Utiger, 1975). Basal TSH measurement is an accepted screening test for congenital hypothyroidism but it has not been used to screen for thyroid dysfunction in adults because present assays cannot reliably identify hyperthyroidism. In clinical practice, therefore, basal TSH measurement has served mainly as a follow-up test to further analyse the cause of low total T_4 concentrations (Figure 1.3).

Basal TSH measurements, by contrast, have a well-established role in the assessment of the adequacy of T_4 replacement therapy, the normalisation of serum TSH being an accepted therapeutic goal which has resulted in a marked reduction in the recommended dosage given (Evered et al., 1973).

Physiological changes in basal TSH levels have been detected using sensitive RIA methods e.g. the diurnal variation of TSH (Wehmann & Nisula, 1984) and the reduction in the basal TSH level due to fasting (Vinik et al., 1975;

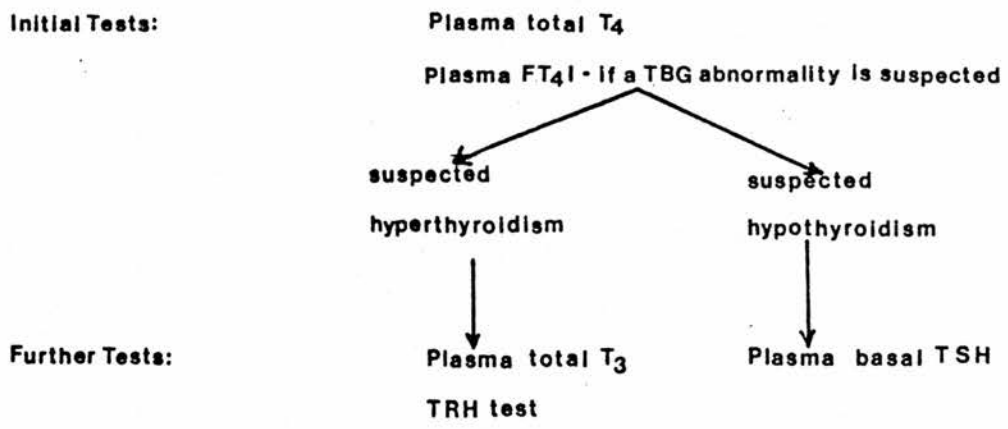


Figure 1.3 A Strategy Commonly Used in the Selection of Biochemical Thyroid Function Tests.

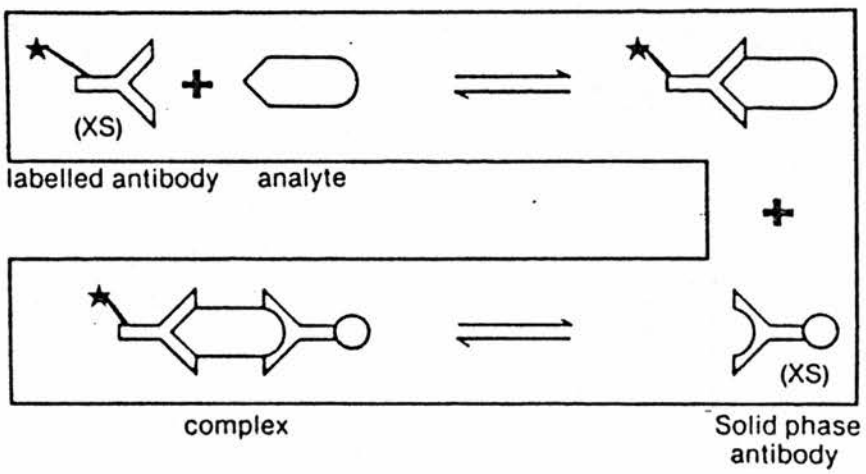


Figure 1.4 A Schematic Representation of the Principle of Immunometric Assay.

The label bound to the solid-phase antibody-analyte complex is directly proportional to the concentration of analyte. Separation of bound from free label is required before quantitation of the bound label.

Croxon et al., 1977). There are conflicting reports as to whether TSH concentrations increase or decrease in pregnancy (Braunstein & Hershman, 1976; Braverman & Vagenakis, 1979; Yamamoto et al., 1979). However, these changes are not sufficient to obscure the rise in basal TSH levels due to primary hypothyroidism.

The development of monoclonal antibodies to TSH has provided reagents for use in labelled-antibody immunoassays (immunometric) for TSH, with the potential for greater sensitivity, specificity and speed of assay (Hunter et al., 1983). The principle of immunometric assay which was proposed by Miles and Hales in 1968 is illustrated in Figure 1.4. In contrast to RIA, an excess of labelled monoclonal antibody is incubated with analyte, and the labelled antibody-analyte complex is removed from solution by an excess of solid-phase antibody. The use of excess reagents enhances the kinetics, reproducibility and hence the sensitivity compared to RIA and the use of two monoclonals, directed to two differing antigenic sites, increases the specificity of the measurement. The first labels used in such assays were radioisotopes (^{125}I) and therefore these assays were termed immunoradiometric assays (IRMA). Fluorescent, chemiluminescent, luminescent and enzyme labels have all since been attached to monoclonal antibodies for immunometric assays. The role of sensitive TSH immunometric measurements in strategies of thyroid function testing has still to be investigated.

1.3.4 The Thyrotrophin-Releasing Hormone Test

The response of the pituitary to the administration of synthetic TRH greatly extended the value of plasma TSH RIA measurements in assessing the function of the HPT axis (Ormston et al., 1971). The test is now performed by injecting 200 μg of TRH intravenously and measuring TSH before and 20 min after the injection. Normally, there is a marked increase in the plasma TSH concentration at 20 min whereas in hyperthyroidism the response is absent due to suppression of the thyrotrophs by high circulating thyroid hormone levels. However, an absent response may also occur when thyroid hormone levels increase within the reference range (subclinical hyperthyroidism) and in patients either receiving T_4 replacement or in remission after treatment for hyperthyroidism (Toft et al., 1981). There is also a temporary failure in the response to TRH during the first 4-8 weeks after treatment of hyperthyroidism due to a delay in the recovery of the previously suppressed thyrotrophs.

Reduced responses may also occur in some non-thyroidal conditions e.g. in patients with Cushing's syndrome, chronic renal failure (Gomez-Pan et al., 1979), depression (Extein et al., 1980), starvation (Carlson et al., 1977) and in some severely ill patients (Heinen et al., 1981; Vierhapper et al., 1982). In most cases, however, the TRH test is thought to provide a reliable

indication of thyroid status (Lamberg & Gordin, 1978; Braverman & Vagenakis, 1979). Drugs known to suppress TSH secretion include dopamine agonists (Morley, 1981) and glucocorticoids (Re et al., 1976). The TRH test is, therefore, of most use in excluding the diagnosis of hyperthyroidism.

In primary hypothyroidism, there is an exaggerated response of TSH to TRH, but this is not generally helpful in diagnosis in view of the raised basal level. In secondary hypothyroidism, basal TSH measurements may be low or normal and there is an impaired response to TRH. However, this condition is usually diagnosed with the help of other pituitary function tests which become abnormal before the TRH test.

Recently, Wide and Dahlberg (1980) have shown with an RIA optimised for high sensitivity, that a good correlation exists between the basal TSH concentration and the magnitude of the TSH response to TRH. Since the TRH test requires personnel to administer TRH and collect two blood samples, and since adverse reactions to TRH have been reported (Editorial, 1984), the possibility of its replacement by a single basal measurement has major advantages. The development of reliable and robust high sensitivity immunometric assays for TSH has made the evaluation of such a replacement, feasible.

1.3.5 Strategies of Thyroid Function Testing

For reasons of economy, most laboratories do not perform both thyroid hormone and TSH measurements on all samples where thyroid function tests have been requested. The shortcoming of TSH RIA methods for detecting hyperthyroidism means that, in general, the strategy shown in Figure 1.3 is commonly used with total T_4 as the first-line test. Such a strategy is used to:-

1. Confirm the presence of thyroid dysfunction.
2. Screen for thyroid dysfunction in the elderly and in the presence of non-thyroidal illness.
3. Monitor the treatment of thyroid disease.

The improvements in methods for free thyroid hormone and TSH measurement may allow the development of more effective strategies of thyroid function testing.

1.4 AIMS OF THE STUDY

The general aims of this study were:-

1. To evaluate the laboratory and clinical performance of analogue fT_4 and fT_3 RIAs and a sensitive TSH IRMA.
2. To ascertain their role in strategies of thyroid function testing.
3. To apply them in situations which might increase understanding of the physiology of the hypothalamic-pituitary-thyroid axis.

Chapter 2

ANALYTICAL METHODS

2.1 MATERIALS

2.1.1 Reagents for Total T_4, T_3 and In-house TSH RIA

All antisera, unless otherwise stated, were from the Scottish Antibody Production Unit (SAPU), Law Hospital, Carluke, U.K. Normal sheep serum (NSS) and normal rabbit serum (NRS) were also from SAPU.

The radiolabels ^{125}I-T_4 and ^{125}I-T_3 (Specific activity (SA) >1200 μCi/μg) were from Amersham International, Amersham, Bucks., U.K. (Amersham International). The ^{125}I-TSH was made by staff at the Immunoassay Section, Department of Clinical Chemistry, Royal Infirmary, Edinburgh, U.K., by a direct chloramine T method (Hunter & Greenwood, 1962).

Solid T_4 (sodium salt pentahydrate) and T_3 (free acid) were from Sigma Chemical Co., Poole, Dorset, U.K. (Sigma) and of >95% quoted purity. The TSH standard was the first WHO International Reference Preparation (IRP) 68/38.

Normal human pooled serum was from outdated donor collections tested for hepatitis B (and more recently, HIV) by the Blood Transfusion Service. Horse serum was from Wellcome Diagnostics, Dartford, Kent, U.K. (Wellcome).

Norit A activated charcoal powder (C5260), triton-X-100 and 8-anilino-1-naphthalene sulphonic acid

(ANS) were from Sigma. All other chemicals were of analytical grade from British Drug Houses Chemicals Ltd., Poole, Dorset, U.K. (BDH).

2.1.2 Thyroid Function Test Kits

Kits used to measure free thyroid hormones were from Amersham International (Amerlex/Amerlex magnetic fT_4 and fT_3), Corning Medical and Scientific, Halstead, Essex, U.K. (Corning Magic fT_4), Becton Dickinson Ltd., Cowley, Oxford, U.K. (Becton Dickinson fT_4, fT_3 and the SimulTRAC fT_4/TSH kit) and Diagnostic Products Ltd., Wallingford, Oxon, U.K. (Coat a Count fT_4 and fT_3). The Riagnost kit for TBG was from Behring Diagnostics, Hounslow, Middlesex, U.K. Kits used to measure TSH were from Becton Dickinson Ltd., (Becton Dickinson ^{125}I-TSH RIA and the SimulTRAC fT_4/TSH Kit), Boots-Celltech Diagnostics Ltd., Slough, Berks., U.K. (Boots-Celltech Sucrosep TSH IRMA) and Amersham International (Amerwell TSH IRMA).

2.1.3 Detection of Abnormal Binding Proteins and the Measurement of Non-esterified Fatty Acids

Radiolabelled thyroid hormones were from Amersham International, agarose gel universal plates from Corning Medical and Scientific and the Wako NEFAC kit for non-esterified fatty acids (NEFA) from Alpha Laboratories, Eastleigh, Hants., U.K. (Alpha Laboratories).

2.1.4 Serum Markers of Thyroid Status

The sex hormone binding globulin (SHBG) kit was from Farmos Diagnostica, General Diagnostics, Science

Park, Cambridge, U.K., and the kit for creatine kinase (CK) was from Randox Laboratories Ltd., Crumlin, Co. Antrim, Northern Ireland. Purified human B_1B_1 glutathione S-transferase (GST), ^{125}I-GST B_1B_1 and rabbit anti-GST B_1B_1 were gifts from Dr J D Hayes, Miss A Hussey and Dr G J Beckett, Department of Clinical Chemistry, Royal Infirmary, Edinburgh, U.K. Microcrystalline cellulose was from Merck, Darmstadt, West Germany, Brij-35 surfactant and other Technicon Clinical Chemistry reagents from Technicon Instruments Co., Basingstoke, Hants., U.K., and furylacryloyl-phenylalanyl-glycylglycine (FAPGG) was from Sigma.

2.1.5. Equipment

Polystyrene tubes (75 x 12 mm) for immunoassay were from Alpha Laboratories. The Microlab-M microprocessor-controlled diluter/dispenser and the cryofuge 6-4 with diagnostic rotor were from V.A. Howe and Co. Ltd., London, U.K. The multiwell gamma counter was an NE1600 from Nuclear Enterprises Ltd., Edinburgh, U.K. The single-well dual channel gamma counter was from LKB Instruments Ltd., Selsdon, S. Croydon, Surrey, U.K., and the Kemtek 3000 was from Kemble Instruments Ltd., Burgess Hill, Sussex, U.K. The sucrose separator was from Boots-Celltech Diagnostics Ltd., and the Amerwell washer from Amersham International. The electrophoresis tank was from Corning-EEL, Halstead, Essex, U.K., the Cobas Bio centrifugal analyser from Roche Diagnostica, Welwyn

Garden City, Herts., U.K., and the Sequential Multiple Analyser plus Computer (SMAC) was from Technicon.

2.1.6 Curve-fitting and Data Processing of Immunoassays

Unless otherwise stated, RIA data processing and curve-fitting was performed using a non-linear 4 parameter log-logistic model (Healy, 1972) using an iterative least-squares technique on a Hewlett Packard 9821 calculator.

The Edinburgh Immunoassay Package (Raab & McKenzie, 1979) which involves a 5 parameter log-logistic curve-fit and the WHO immunoassay program based on a mass action model (Edwards & Ekins, 1983) were used in the data processing of TSH IRMA counts with a Superbrain and an Apple II microcomputer, respectively.

2.2 THYROID FUNCTION TESTS

2.2.1 Assays for Total Thyroid Hormones

(a) Preparation of standards in charcoal-stripped serum

Iodothyronines were removed from serum by mixing with Norit A activated charcoal (100 g/l) at room temperature for 4 h. To assess the effectiveness of this procedure, radiolabelled T_4 was pre-incubated for 1 h with the serum before charcoal treatment. The charcoal was removed by centrifugation at 30,000 g on a MSE 18 high speed centrifuge (20 min) followed by filtration of the serum through two Sartorius membrane filters (0.45 μ and 0.2 μ) under reduced pressure. Radioactive counting showed that >98% of T_4 was removed by this process.

Concentrations of total protein and albumin were not changed significantly by this procedure.

Stock solutions of T_4 and T_3 were made by dissolving 20 mg of T_4 sodium salt in 50% polyethylene glycol (PEG) (20 ml) made alkaline with a few drops of ethanolamine and by dissolving 10 mg of T_3 (free acid) in methanol (10 ml) rendered alkaline with concentrated NaOH. The accuracy of these solutions was assessed spectrophotometrically by diluting the stocks (0.2 ml) with freshly prepared 0.04 M sodium hydroxide (2 ml) and measuring the absorbance at wavelengths of 325 nm (T_4) and 320 nm (T_3) against appropriate solvent blanks (Malan et al., 1983). The concentration (C) was calculated from the equation:

$$C = \frac{\text{Absorbance x Dilution factor}}{\text{Pathlength x } \varepsilon}$$

where ε is the molar extinction coefficient taken as 6200 and 4660 l/mol.cm for T_4 and T_3 respectively (Gemmil, 1955; Edelhoch, 1962). If the agreement between the spectrophotometric values and those calculated gravimetrically was within 2%, the stock solutions were diluted serially in charcoal-stripped human serum to give working standards: T_4, 25-300 nmol/l; T_3, 0.5-10 nmol/l. These were stable at -20°C for at least 6 months.

(b) Radioimmunoassay procedures

Staff of this Clinical Chemistry Department measured total T_4 and T_3 for the routine investigations of patients attending the thyroid clinic (Chapters 4 & 7); all other assays were performed by myself.

The RIA procedure was based on the method of Ratcliffe et al. (1974) using 0.05 M barbitone buffer pH 8.6 containing 0.1% gelatine and 0.01% thiomersal, as diluent. Primary sheep antibody, tracer (radiolabel) and standard or sample were incubated in duplicate with ANS to block the binding of T_4 or T_3 to serum protein-binding sites but with minimal effect on antibody binding. The antibody-bound fraction was separated from the free radiolabel by precipitation with donkey anti-sheep serum (DAS) with the addition of carrier immunoglobulins (NSS) to permit cross-linking of the immune complexes and precipitate formation. These double-antibody reagents were included in the RIA incubates and, for complete precipitation, assays were incubated overnight at 4°C followed by centrifugation at 4°C for 30 min at 2,000 g. The procedures for RIA of total T_4 and T_3 were similar and are summarised below:

Total T_4	Total T_3
20 µl sample/standard	50 µl sample/standard
600 µl tracer reagent (DAS, ^{125}I-T_4)	660 µl tracer reagent (DAS, ^{125}I-T_3, ANS)
200 µl primary antibody reagent (Anti-T_4, NSS, ANS)	300 µl primary antibody reagent (Anti-T_3, NSS)

Vortex
Incubate 4°C overnight
Centrifuge 2,000 g, 30 min, 4°C
Decant
Count pellets

Samples were dispensed with tracer/DAS reagent followed by the repeat dispensing of primary antibody reagent.

(c) Optimisation of the methods

Optimal precipitating dilutions of DAS and NSS used in the double-antibody separation were determined by using a matrix of DAS/NSS combinations to separate zero standard RIA incubates (B_o) containing primary antibody, tracer and ANS. The results of such an experiment are shown in Table 2.1 for the precipitation of tracer bound to anti-T_4 antiserum used at 1:10,000 final dilution (FD), as recommended by SAPU. The combination giving the greatest precipitation (1:80 DAS, 1:3300 NSS) on the plateau region outlined in Table 2.1 was chosen for the T_4 assay. Optimal conditions were slightly different for the total T_3 assay, Table 2.2.

Table 2.1 Optimisation of Double-Antibody Precipitation of Antibody-Bound ^{125}I-T_4

% Tracer Bound	Final NSS Dilution				
Final DAS Dilution	x825	x1650	x3300	x6600	x13200
x40	55%	54%	53%	53%	51%
x60	58%	59%	59%	58%	56%
x80	59%	60%	62%	62%	59%
x100	58%	60%	62%	58%	58%
x120	54%	56%	58%	58%	57%

Table 2.2 Final Conditions for Total T_4 and T_3 Assays

Final Dilution	T_4	T_3
Primary antibody	1: 7,000	1: 15,000
DAS	1: 80	1: 70
NSS	1: 3,300	1: 2,000
ANS	320 µg/tube	100 µg/tube
Radiolabel	20 pg/tube	15 pg/tube

The primary antibody final dilutions were optimised by the use of precision profiles generated from the duplicates of standards and samples (Ekins, 1983b). The dilutions giving the best profiles and widest working ranges at values of most clinical interest were chosen. Working assay reagents were:-

T_4 primary antibody reagent: Diluent (40 ml), Anti-T_4 (24 µl) NSS (50 µl) ANS (64 mg)

T_4 tracer reagent: Diluent (115 ml), DAS (2 ml), ^{125}I-T_4 (100 µl)

T_3 primary antibody reagent: Diluent (210 ml), Anti-T_3 (47 µl) NSS(350 µl)

T_3 tracer reagent: Diluent (150 ml), DAS (3.2 ml), ^{125}I-T_3 (200 µl) ANS (23 mg)

(d) The precision and bias of the total T_4 and T_3 methods

The mean within-assay precision profiles for the total T_4 and T_3 assays calculated from the duplicates of samples are shown in Figure 2.1. The laboratory

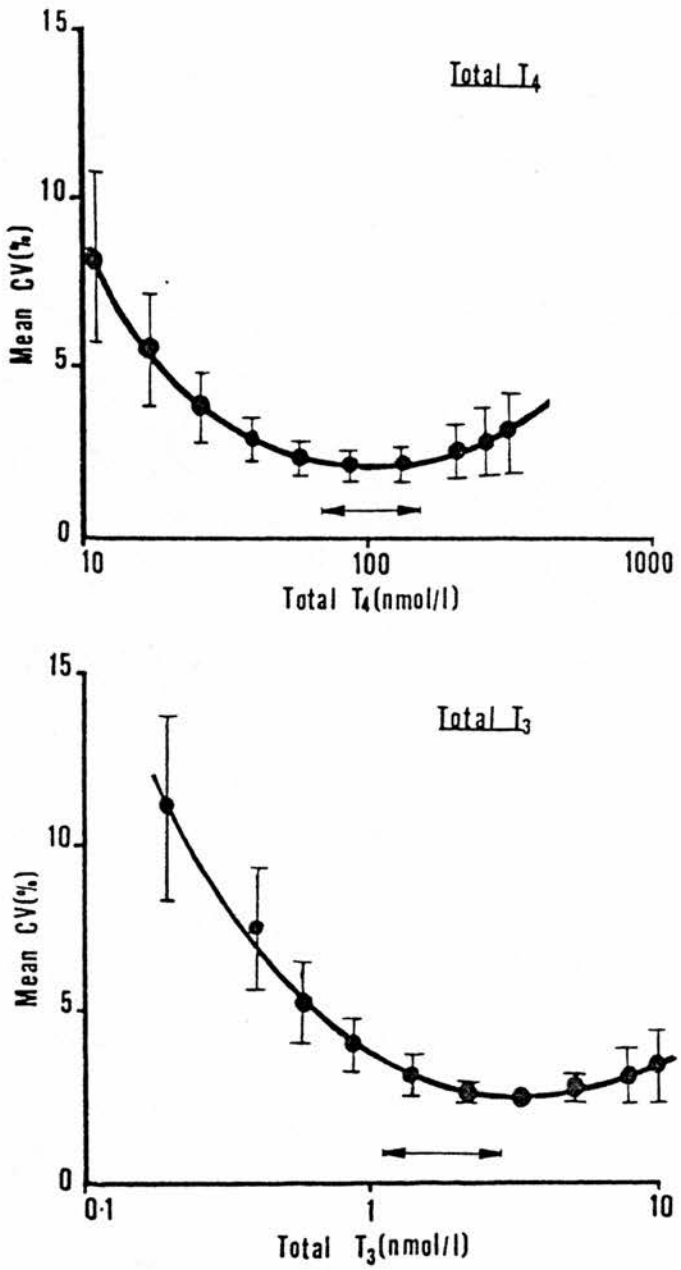


Figure 2.1 Total Thyroid Hormone RIA Within-assay Precision Profiles (Mean ± SD, n = 10).

Reference ranges are shown (◄——►).

reference ranges (T_4 60-150 nmol/l, T_3 1.1-2.8 nmol/l) were derived from normal volunteers and from patients attending a thyroid clinic who were established as being euthyroid by clinical examination and the results of other thyroid function tests (Seth et al., 1976).

Quality control pools used to assess between-assay precision were normal human serum either untreated (medium pool), diluted with charcoal-stripped serum (low pool) or containing added T_4 or T_3 (high pool). These were stored in aliquots at -20°C. In addition, 2 or 3 patient samples were reassayed subsequently (repeat analysis control, RAC) and the results were used to calculate between-assay precision (Table 2.3).

2.2.2 Free Thyroid Hormones by Analogue RIA

The principle of the measurement of free thyroid hormones by analogue RIA is now common to many commercial kits (Figure 1.2b). The assays involve the incubation of sample or standard with solid-phase antibody (solid particles or coated-tube) and a ^{125}I-T_4 or ^{125}I-T_3 derivative (manufacturer-specific) which binds to the assay antibody but which is chemically modified to inhibit its binding to serum proteins. A detailed evaluation of several fT_4 and fT_3 kits is given in Chapter 4.

Table 2.3 Between-assay Precision of Thyroid Function Tests

	QC Pools				RAC						
							Mean				
Test	n	Mean	SD	CV(%)	n	Range	x_1	x_2	t-test	SD	CV(%)
*Total T_4 (nmol/l)	50	47	2.8	6.0	22	25-75	61.9	61.3	NS	4.1	6.7
	51	103	4.1	4.0	54	76-150	114.6	113.6	NS	4.9	4.3
	51	188	7.1	3.8	20	151-300	176.0	180.5	NS	7.1	3.9
*Total T_3 (nmol/l)	37	1.5	0.15	10.0	17	0.5-2.0	1.3	1.3	NS	0.13	9.8
	37	2.2	0.14	6.3	20	2.1-4.0	2.8	2.8	NS	0.12	4.2
	37	6.1	0.29	4.7							
TBG (mg/l)	9	19.5	0.7	3.7	35	15.0-35.0	24.2	23.7	NS	1.1	4.8
*TSH RIA (mU/l)	34	2.9	0.2	6.8	26	<2.5	1.7	1.6	NS	0.21	12.9
	34	7.8	0.6	7.8	19	2.5-5.0	3.6	3.8	p<0.05	0.28	7.7
	35	21.6	1.5	6.8	21	5.0-10.0	6.9	6.9	NS	1.68	9.8
	35	46.8	3.4	7.2	19	10.0-20.0	16.0	16.1	NS	1.27	7.9
					28	20.0-40.0	27.5	27.3	NS	2.41	8.8
					26	>40.0	55.9	52.8	p<0.005	4.40	8.1

* Combined data from assays performed by myself and the routine diagnostic service.
NS = Not significant.

2.2.3 Measurement of Thyroxine Binding Globulin (TBG)

The freeze-dried kit reagents and standards were reconstituted in distilled water and the RIA performed as described in the package insert and summarised below:-

20 µl sample/standard/control
200 µl ^{125}I-TBG
Vortex
200 µl Anti-TBG
Vortex
Incubate 2-4 h, room temperature
Add 1 ml PEG solution
Vortex
Centrifuge 20 min, >1500 g
Decant supernatant
Count pellets

A control serum was supplied with each kit and the mean bias from the quoted value in 9 assays was +5.7 ±1.9%. A mean within-assay precision profile is illustrated in Figure 2.2 and the between-assay precision data is shown in Table 2.3. At present there is no international reference standard for TBG. The manufacturer's recommended reference ranges derived from 368 healthy adults aged 17-60 years were used (males: 8.7-30.7, females: 10.5-31.7 mg/l).

2.2.4 Measurement of TSH by In-house RIA

Staff at the Immunoassay Section of this Clinical Chemistry Department measured TSH for the routine investigation of new patients attending the thyroid clinic. The RIA used was an in-house double-antibody method (Toft et al., 1973) using 0.05 M phosphate buffer pH 7.5, containing 2% horse serum, 0.05% triton -X-100 and 0.02%

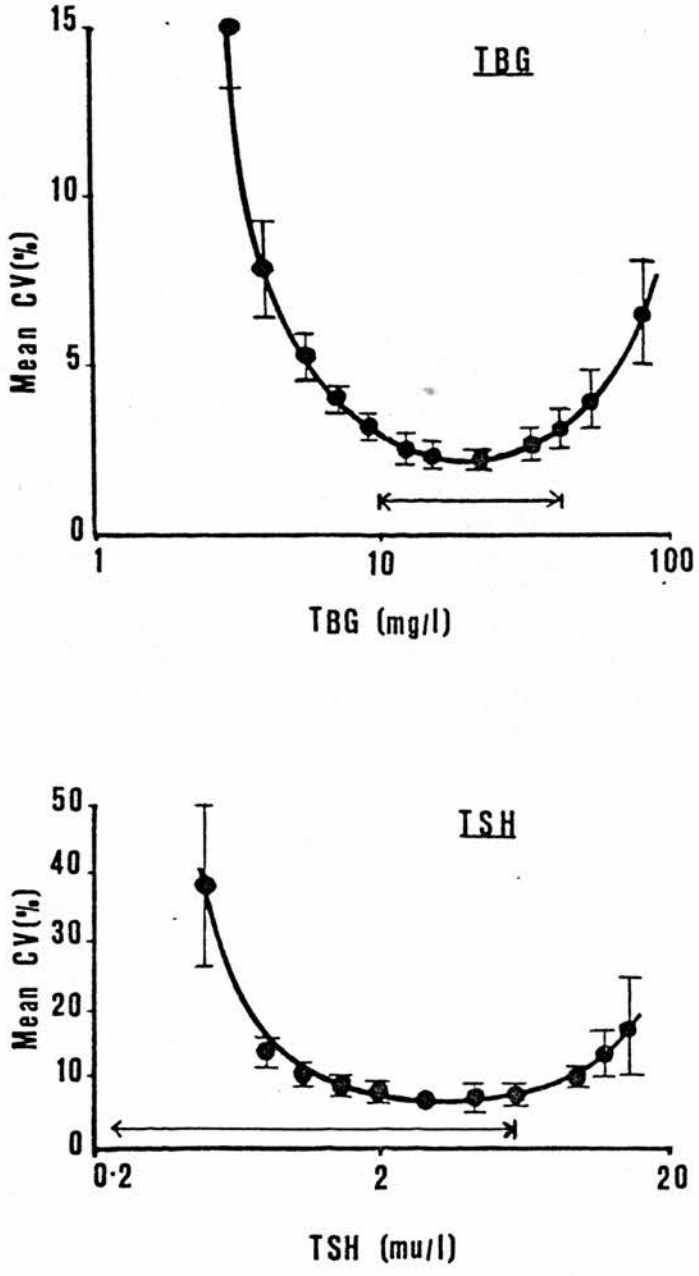


Figure 2.2 Within-assay Precision Profiles (Mean ± SD) for TBG (n = 8) and the In-house TSH (n = 12) Assays.

Reference Ranges (⟷).

sodium azide as assay diluent. Standards in assay diluent (0.46-14.8 mU/l) and samples (100 µl) were incubated in duplicate with rabbit anti-TSH (50 µl, FD 1:200,000) and assay diluent (200 µl) for 24 h at room temperature. This antiserum was from Professor Butt, Birmingham and Midland Hospital for Women, Birmingham, U.K. The TSH tracer was cleaned of inorganic iodide and TSH aggregates before use by gel filtration (G10/G150 Sephadex) and diluted to 1.5 ng/ml for use. After adding NRS to the tracer solution, 50 µl was added to RIA incubates (FD NRS 1:9,000) for the second incubation of 24 h at room temperature. Donkey anti-rabbit serum (DARS) in diluent containing 9 mmol/l EDTA (50 µl, FD 1:450) was then added to precipitate the bound fraction, overnight at 4°C. The Kemtek 3000 was used to filter, wash and count the precipitated fraction of tracer. Data processing was with a 4-parameter logistic curve fit.

The mean within-assay precision profile and typical between-assay precision data for this assay are shown in Figure 2.2 and Table 2.3, respectively. The mean 6 month bias from the all-method mean in the Birmingham External Quality Assessment Scheme (EQAS) was typically +3.6% ± 10.6. The mean recovery of the method assessed in 3 recovery experiments by EQAS was 93% ± 13 compared with the all-method mean recovery of 97% ± 6.

The upper reference limit for TSH by this method (5.7 mU/l) was derived from euthyroid patients attending a thyroid clinic (Toft et al., 1973) and the normal range of TSH response 20 min after TRH injection (200 μg) was 3.9 -25.3 mU/l (Toft et al., 1978).

2.2.5 Measurement of TSH by Commercial Assay

Details of the analytical and diagnostic performance of the various commercial RIA and IRMA methods for TSH are given in Chapter 4. In the Becton Dickinson single and dual-analyte RIA kits, there were two incubation options. In this thesis, the 4 h high sensitivity option which omits the top standard supplied, was evaluated for both kits.

2.3 THE DETECTION OF ABNORMAL BINDING PROTEINS

2.3.1 A Screening Test for Anti-T_4 or T_3 Autoantibodies

Samples were screened initially for the presence of circulating anti-T_4 and anti-T_3 antibodies by comparison of ^{125}I-T_4 or ^{125}I-T_3 binding by patient and normal sera. Sera (10 μl) were incubated with 200 μl 0.05 M barbitone buffer pH 8.6 containing 7 fmol ^{125}I-labelled T_4 or T_3 for >2 h at room temperature. Gamma globulin-bound radiolabel was precipitated by the addition of 50% saturated ammonium sulphate solution (3 ml) followed by centrifugation at 2,000 g for 15 min. The percentage of total counts in the pellet was generally <5% in normal sera.

2.3.2 Measurement of the Distribution of Thyroid Hormone Binding to Serum Proteins

The patient's serum (50 µl) was incubated at room temperature for 2 h with ^{125}I-labelled T_4 or T_3 (70 fmol). The proteins in 1 µl of serum were then separated by agarose electrophoresis. Electrophoresis buffer was 0.05 M pH 8.6 sodium barbitone containing 0.035% EDTA. Samples were run in triplicate for 30 min and after electrophoresis, one sample was stained for protein with amido black (5% acetic acid destain) whilst the other samples were sliced into 0.5 cm strips and counted for ^{125}I-radioactivity (T_4 and T_3 binding). A control sample with no known abnormality in binding was electrophoresed in triplicate with each test serum. The percentage distribution of T_3 or T_4 between the serum proteins was calculated from the total counts of the electrophoresis segments (Beckett et al., 1983b).

2.4 THE MEASUREMENT OF NON-ESTERIFIED FATTY ACIDS

Serum NEFA were measured by the Wako enzymatic method with the colorimetric endpoint monitored at 550 nm using the Cobas Bio Analyser. The principle of the test is summarised below:-

$$\underset{\text{(NEFA)}}{\text{RCOOH}} + \text{ATP} + \underset{\text{(CoA)}}{\text{Coenzyme A}} \xrightarrow{\text{ACS}} \text{AcylCoA} + \text{AMP} + \text{PPi}$$

$$\text{AcylCoA} + O_2 \xrightarrow{\text{ACOD}} \text{2,3 trans-EnoylCoA} + H_2O_2$$

3-methyl-N-ethyl-N-(hydroxyethyl)-aniline (MEHA)

+

$$\text{4-aminoantipyrine} \xrightarrow[\text{POD}]{H_2O_2} \underset{\text{Product}}{\text{Purple Condensation}} + 4\ H_2O$$

(ACS = AcylCoA synthetase; ACOD = AcylCoA oxidase; POD = Peroxidase)

The assay was calibrated with 1 mM oleic acid and adapted for use on the Cobas Bio as follows to give the recommended final reaction conditions:-

Colour reagent A (ACS, Ascorbate oxidase, CoA, ATP, 4-aminoantipyrine) was reconstituted in 10 ml of supplied solvent A as described for the manual method. Colour reagent B (ACOD, POD, MEHA) was reconstituted in 2.5 ml of supplied solvent B. Sample (10 µl) was preincubated with reagent A (200 µl) at 37°C for 10 min then reagent B (50 µl) was added as start reagent and 17 absorbance readings were taken at 20 s intervals. Serum samples were used for assay, where possible, since heparin is known to

cause an increase of 10% in NEFA values (Knox & Jones, 1984). The within-rotor reproducibility (n = 28) and the between-assay precision (n = 10) were estimated as 3.1% and 6.2% respectively.

NEFA values in serum increased two-fold if left at room temperature for 48 h whereas little change occurred at 4°C for 48 h and samples stored at -20°C were stable for at least four weeks. The manufacturer's expected reference range for fasting patients is 0.1 - 0.6 mmol/l.

2.5 MEASUREMENT OF SERUM MARKERS OF THYROID STATUS

The between-assay data for the following methods are shown in Table 2.4.

2.5.1 Assay of Sex Hormone Binding Globulin (SHBG)

The commercial method for measurement of SHBG is based on a liquid-phase IRMA (Hammond et al., 1985). Samples and standards were diluted 100-fold in assay buffer and 100 µl aliquots incubated with 200 µl of anti-SHBG polyclonal antiserum and ^{125}I-anti-SHBG monoclonal antibody at room temperature for 1 h. A solid-phase second antibody was then added, followed 15 min later by 2 ml 0.9% NaCl. After centrifugation (2,000 g 15 min), the supernatants were decanted to waste. Curve-fitting and data interpolation were performed manually using linear-linear graph paper. Over the range 7-110 nmol/l SHBG, the within-assay precision ranged from 1.8 to 4.9%. In 10 samples issued by the SHBG EQAS (Clinical Chemistry,

Table 2.4. The Precision of Assays for Serum Markers of Thyroid Status

	QC Pools				RAC						
Assay	n	Mean	SD	CV	n	Range	Mean x_1	x_2	SD	t-test	CV
SHBG	8	94	9.6	10.2%	22	27-110	59.8	57.3	5.3	NS	9.1%
GST	15	2.5	0.2	8.8%	10	2.5-8.5	5.75	5.71	0.3	NS	5.2%
(µg/l)	36	5.8	0.5	9.4%							
ACE	11	83	2.1	2.5%	40	38-112	74.1	77.5	4.6	p<0.005	6.1%
(U/l)	17	138	5.9	4.3%							
CK	10	283	4.8	1.7%	*44	<400	140.9	140.0	4.6	NS	3.3%
(U/l)	10	488	5.9	1.2%	*34	>400	879.9	876.6	15.9	NS	1.9%
* ALT	23	61	6.3	10.3%	442	10-50	23.2	22.4	2.7	p<0.0001	11.7%
(U/l)					52	51-100	71.3	68.8	3.0	p<0.0001	4.3%
					28	101-500	167.0	164.4	4.7	NS	2.8%
*GGT	24	124	2.5	2.0%	566	<200	38.0	37.8	1.7	NS	4.5%
(U/l)											
*Creatinine	23	392	5.1	1.3%	552	<200	99.0	99.8	3.6	p<0.0001	3.6%
(µmol/l)					80	201-900	428.9	429.6	6.0	NS	1.4%

*Data from the routine diagnostic service NS = Not significant

City Hospital, Nottingham, U.K.) the mean bias was -0.2% ± 8.3%. The reference ranges from the kit insert were used in this thesis: males 10-50, females 30-90 nmol/l.

2.5.2 Assay of Glutathione S-Transferase (GST)

The human liver-specific (B_1B_1) form of GST was measured in serum by the RIA method of Beckett and Hayes, 1984. The assay employed a delayed addition of tracer followed by double-antibody separation. Assay diluent was 0.05 M potassium phosphate, pH 7.5, containing bovine serum albumin (1 g/l) and sodium azide (0.2 g/l). Standards (purified GST B_1B_1 enzyme) were made up in assay diluent and stored at -20°C. Standards and samples (100 µl) were incubated in duplicate with primary antibody/NRS reagent (100 µl; FD 1:38,000 anti-GST B_1B_1 and 1:1,000 NRS) for 24 h 4°C. Radiolabelled GST B_1B_1 in assay diluent (50 µl; 20,000 cpm) was then added and the tubes incubated at 4°C for a further 24 h. Precipitating antiserum (DARS) was then added (100 µl; final dilution 1:105) and the tubes incubated at 4°C for a further 16 h. A wash solution (2 ml) of 0.01% microcrystalline cellulose and 0.1% Brij in distilled water was added to incubates prior to centrifugation at 2000 g for 30 min at 4°C. Supernatants were decanted and the pellets counted in the NE1600 gamma counter. A mean within-assay precision profile for patient samples is shown in Figure 2.3. The upper reference limit for the assay of 4.0 µg/l was determined

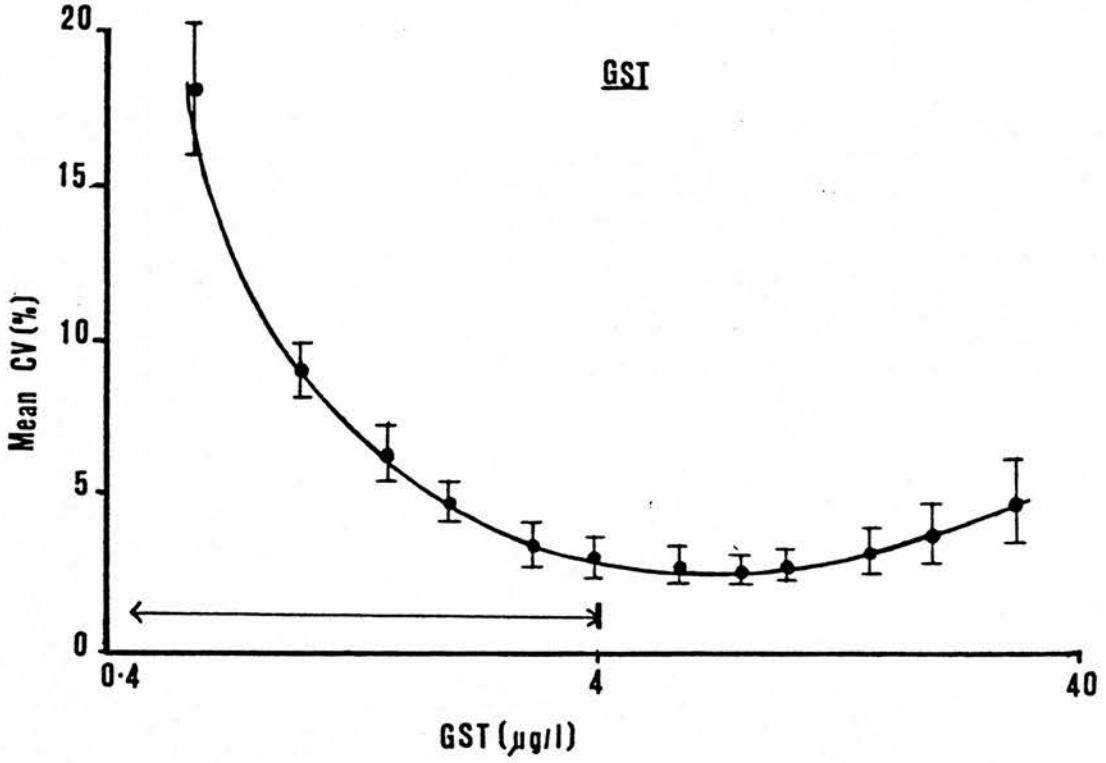


Figure 2.3 The Within-assay Precision Profile for GST RIA (Mean ± SD, n=8).

Reference Range (⟷).

from 135 blood donors and 40 laboratory volunteers (Beckett et al., 1985).

2.5.3 Assay of Angiotensin-Converting Enzyme (ACE)

The concentration of ACE (EC 3.4.15.1) in serum was determined by continuous spectrophotometric monitoring of the hydrolysis of FAPGG to furyl-acrylyl phenylalanine and glycylglycine at 37°C based on the method of Maguire and Price, 1985. The decrease in absorbance at 344 nm was monitored on a Cobas Bio centrifugal analyser using a reagent blank. The assay buffer was 0.05 M Tris containing 0.4 M sodium chloride, pH 8.3 at room temperature. The substrate was 2 mmol/l FAPGG in assay buffer and the volumes added to the reaction cuvette were: 30 µl serum sample, 20 µl assay buffer and 50 µl substrate reagent. Absorbance readings were made after 8 min and at 30 s intervals thereafter for 7 min. The within rotor reproducibility of the method was 3.9%. The assay was linear up to values of 200 U/l. The enzyme activity in U/l was calculated by multiplication of the absorbance change per minute by the conversion factor 5367 (derived from the molar extinction coefficient, pathlength and sample dilution in the cuvette). The reference range for serum ACE (9-109 U/l) was derived from 100 surgical in-patients with no known condition which would cause raised ACE levels e.g. sarcoid, diabetes mellitus or hyperthyroidism.

2.5.4 Assay of Creatine Kinase (CK)

Creatine kinase (EC 2.7.3.2) in serum was measured by the optimised standard method at 37°C using the following coupled reaction system:

$$\text{Creatine phosphate} + \text{ADP} \xrightarrow{\text{CK}} \text{Creatine} + \text{ATP}$$

$$\text{Glucose} + \text{ATP} \xrightarrow{\text{HK}} \text{Glucose-6-P} + \text{ADP}$$

$$\text{Glucose-6-P} + \text{NADP}^+ \xrightarrow{\text{G6P-DH}} \text{6-phosphogluconic acid} + \text{NADPH} + \text{H}^+$$

(HK = Hexokinase, G6P-DH = Glucose-6-P dehydrogenase)

The increase in absorbance at 340 nm was monitored on a Cobas Bio analyser with the first reading 80 s after mixing followed by nine readings at 20 s intervals. The volumes used in the reaction mixture were; 5 µl sample, 20 µl diluent and 200 µl reagent. The conversion factor to U/l was 5358. The laboratory reference ranges for this assay were: males 30 - 200, females 30 - 150 U/l.

2.5.5 Other Clinical Chemistry Analyses

Alanine aminotransferase (ALT) (EC 2.6.1.2), gamma-glutamyltransferase (GGT) (EC 2.3.2.2) and creatinine were measured in serum by standard methods on a SMAC II analyser. The laboratory reference ranges for these analytes were: ALT, 10-40 U/l; GGT, 10-55 U/l males and 5-35 U/l females; creatinine, 55-150 µmol/l. The

principles of the methods were:-

ALT: measurement of the decrease in absorbance at 340 nm.

$$\text{Alanine} + \text{ketoglutarate} \xrightarrow{\text{ALT}} \text{Pyruvate} + \text{glutamate}$$

$$\text{Pyruvate} + \text{NADH} \xrightarrow{\text{LDH}} \text{NAD} + \text{lactate}$$

(LDH = lactate dehydrogenase)

GGT: measurement of the increase in absorbance at 410 nm.

$$\gamma\text{-glutamyl p-nitroanilide} + \text{glycylglycine} \xrightarrow{\text{GGT}} \gamma\text{-glutamylglycylglycine} + \text{p-nitroaniline}$$

Creatinine: measurement of the absorbance at 505 nm.

$$\text{creatinine} + \text{picric acid} \xrightarrow{OH^-} \text{Creatinine-alkaline picrate complex (Red Chromogen)}$$

Chapter 3

DEVELOPMENT OF EQUILIBRIUM DIALYSIS REFERENCE METHODS FOR FREE THYROID HORMONES

In this chapter the experiments performed to develop sensitive RIA methods for T_4 and T_3 are described. In addition, various factors which affect the free thyroid hormone levels as measured by equilibrium dialysis-RIA have been investigated.

3.1 MATERIALS

3.1.1 Radioimmunoassay Reagents

Primary sheep antisera to T_4 were from (a) SAPU (batch no. 056G) (b) the RAST Allergy Unit, Benenden Chest Hospital, Cranbrook, Kent, U.K. (Code ST4H H/38) (c) Advanced Laboratory Techniques, Tunbridge Wells, Kent, U.K. (ALS) (d) Amersham International (personal gift). The high-titre sheep anti-T_3 antiserum was also from Amersham International. Soluble double-antibody reagents were from SAPU, SacCel anti-sheep cellulose-coupled antibody from Wellcome and Sephacryl-linked SAPU anti-sheep antibody from Dr J Wright, MRC Immunoassay Section, Edinburgh, U.K. ^{125}I-T_4 and ^{125}I-T_3 were from Amersham International, reverse T_3 (L-3,3',5' Tri-iodothyronine) from Cambridge Bioscience, Cambridge, U.K., and other thyroid hormone solids were from Sigma.

3.1.2 General Materials and Equipment

HEPES (N-2-hydroxyethylpiperazine-N'-2-ethane-sulfonic acid), gentamicin sulphate and bovine gamma globulin (G-5009) were from Sigma. All other reagents were from BDH. The diluent for RIA was 0.01M HEPES containing

106 mM sodium chloride and 1 mM sodium azide, pH 7.4 at 37°C.

Dialysis membranes were Visking tubing 32/32 from Scientific Instrument Centre Ltd., London, U.K., and Spectrapor membrane (No.2) from Spectrum Medical Industries Inc., Los Angeles, U.S.A. The equilibrium dialysis system was from Dianorm, Fisons Ltd., Crawley, Sussex, U.K., with paired teflon macro half-cells (1.2 ml volume each) and the water bath with immersion heater was from Grant Instruments, Cambridge, U.K.

3.2 CHOICE OF SEPARATION SYSTEM FOR RIA

Various non-specific methods for separating bound and free ligand have been used in the RIA of free thyroid hormones in serum dialysates e.g. dextran-coated charcoal adsorption (Ellis & Ekins, 1975; Jiang & Tue, 1977; Romelli & Pennisi 1979), paper chromatography (Weeke & Orskov, 1979) and, more recently, precipitation of the protein-bound ligand with polyethylene glycol (PEG) (Helenius & Liewendahl, 1983). In this thesis, a specific separation system was developed and compared with PEG precipitation.

The use of first antibody coupled to microcrystalline cellulose particles (Seth et al., 1976) was tried, initially. This system was not investigated further due to the following disadvantages:-

(a) Cyanogen bromide was used to activate the cellulose.

(b) A fall of at least 50% in antibody titre after coupling to the cellulose was observed as described previously (Wide, 1969).

(c) An overnight incubation was required for equilibrium to be reached in the RIA.

(d) Experience with the SAPU anti-T_4 antiserum showed that in solid-phase, the antibody binding showed a marked pH optimum of pH 10.5 , making the measurement of fT_4 in dialysates which had a pH of 7.4 a problem.

Specific separation by a soluble double-antibody precipitation method was described for the measurement of total T_4 and T_3 in Chapter 2. Modifications of this technique include:-

1. Covalent coupling of DAS to an insoluble matrix used to separate primary antibody-antigen complexes.
2. Post-precipitation of soluble antisera i.e. primary antibody, analyte and radiolabel incubated in the presence of DAS and NSS (cf. total T_4 and T_3 RIA).
3. Pre-precipitation of NSS with DAS to form a non-covalent solid-phase which may be used to separate primary antibody-antigen complexes (Brown et al., 1980).
4. Pre-precipitation of the primary antibody with DAS and NSS. This forms a non-covalent solid-phase which is added to RIA incubates (Hales & Randle, 1963).

These variations of the double-antibody technique were investigated in the development of a high sensitivity assay for T_4 and then applied to the measurement of T_3.

3.2.1 T_4 RIA Incubation Mixtures

Details of the procedures used to optimise the RIA and to prepare stable T_4 standards (3-100 pmol/l) are described in subsequent sections of this chapter. In the evaluation of the separation systems described below, standards in HEPES buffer pH 7.4 with 0.3% gelatine (250 µl) were incubated with RAST primary antiserum (40 µl; FD 1:200,000 and 3.5 fmol ^{125}I-T_4 (40 µl). When this antibody was used in solution, equilibrium for the primary binding reaction was reached after 3 h at room temperature, after which the various separation methods were employed.

3.2.2 Separation by PEG Precipitation

The concentrations of added carrier protein (bovine gamma globulin, 100 µl) and PEG (1.5 ml) which gave maximum precipitation of antibody-bound radiolabel in the absence of antigen (B_o), but minimum precipitation of radiolabel in the absence of primary antibody (non-specific binding, NSB), were determined by adding different concentrations of these reagents to B_o and NSB incubates (Table 3.1). The concentrations of 20 g/l gamma globulin and 300 g/l PEG in HEPES buffer were used in subsequent assays. A minimum incubation time of

Table 3.1 Optimisation of Reagent Concentrations for PEG Separation

PEG (g/l)	Bo (NSB)*				
	100	200	250	300	350
γglobulin (g/l)					
5	8.5(3.5)	38.5(6.2)	53.4(6.0)	60.0(7.1)	61.7(7.9)
10	10.4(8.4)	45.7(9.0)	63.9(9.5)	62.3(11.5)	66.7(13.1)
20	13.4(7.0)	54.9(8.4)	64.7(10.2)	67.1(10.9)	70.8(15.5)
30	8.1(9.2)	57.6(8.7)	64.6(9.6)	67.0(11.6)	70.4(17.0)
40	7.7(11.7)	56.8(18.6)	68.0(15.3)	68.5(16.2)	72.7(21.0)

*NSB values are in brackets and expressed as the % of total counts added.

Values representing a plateau region are outlined.

15 min at room temperature with PEG was necessary for complete precipitation of protein. Incubates were then centrifuged for 15 min at 2,000 g and the radioactivity in the protein pellets counted. Re-suspension of pellets in 300 g/l PEG solution and centrifugation was necessary to reduce NSB values to <5%.

3.2.3 Separating Antibody Covalently Linked to Solid-Phase

The SAPU donkey anti-sheep antiserum linked to sephacryl (Sephacryl-DAS) was made by coupling either the whole antiserum or the IgG fraction only to sephacryl, by the method of Wright and Hunter, 1982. Stock solutions were stored at a ten-fold dilution of the original antiserum at 4°C. A commercial anti-sheep antibody linked to microcrystalline cellulose (SacCel) was also tried. Separation was effected by addition of optimal concentrations of these reagents to RIA incubates i.e. 1:20 dilution of SacCel (100 µl) or a 1:10 dilution of Sephacryl-DAS (100 µl) with incubation at room temperature for 30 min (SacCel) or 1 h with shaking (Sephacryl-DAS). A distilled water wash was added to the tubes prior to centrifugation at 2,000 g for 15 min.

The optimal dilutions of the separating reagents described above were determined by using increasing dilutions of stock solutions to separate bound and free ^{125}I-T_4 in a series of B_o incubates. The SacCel and IgG Sephacryl-DAS produced maximum precipitation of primary

antibody up to a 30-fold dilution of stock reagents, whereas with Sephacryl-DAS made from whole serum, the precipitation of bound tracer declined rapidly at dilutions greater than ten-fold. Use of SacCel at 1:20 and Sephacryl-DAS at 1:10 dilutions of stock produced maximum B_o values but re-suspension of the pellet with a further 1 ml wash and re-centrifugation was necessary, to achieve NSB values of <5%.

3.2.4 Post-precipitation Double-Antibody Separation

Optimal final dilutions of DAS (1:80) and NSS (1:3,300) for precipitation were as determined previously for the total serum T_4 method (Table 2.1). An overnight incubation at 4°C was required for complete precipitation and this was followed by centrifugation at 4°C (2,000 g) for 30 min to sediment the precipitate.

The use of this method resulted in <10% binding of tracer and increasing the amount of RAST anti-T_4 antibody present from 1:2,000,000 to 1:200,000 FD produced little improvement in tracer binding (Figure 3.1). The removal of endogenous iodothyronines in these reagents by pre-treatment of the DAS and NSS with charcoal (Section 2.2.1) also produced no significant increase in binding. A sensitive RIA for T_4 could not, therefore, be developed using this method. In an attempt to remove the interference found using the whole animal sera (DAS and NSS) in RIA incubates, DAS was pre-precipitated with either NSS alone or with NSS and anti-T_4 antibody.

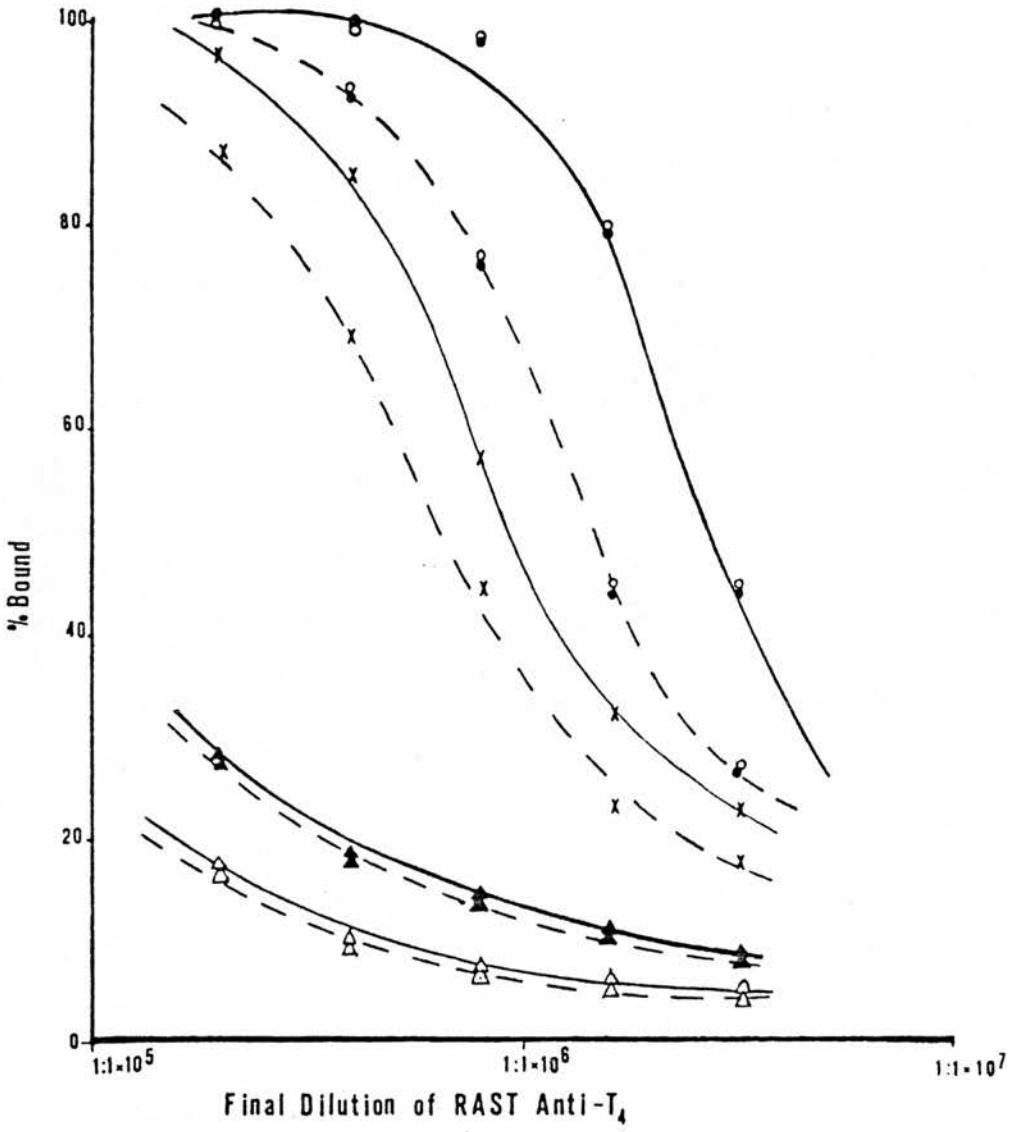


Figure 3.1 RAST Anti-T4 Dilution Curves with Zero (——) and 50 pmol/l Added T4 (- - -).

Separation of bound and free ligand was by double-antibody post-precipitation using untreated (Δ) and charcoal-stripped (▲) reagents or by a preformed DAS-NSS precipitate with untreated (o) and charcoal-stripped (●) DAS and NSS added at the end of the primary reaction.
Dilution curves using an unwashed DAS-NSS precipitate are also shown (x).

3.2.5 Pre-precipitation of Separating Antibody with Normal Sheep Serum

As an alternative to the post-precipitation method described in Section 3.2.4, the possibility of using a pre-formed DAS-NSS precipitate in a similar way to the use of SacCel at the end of the primary reaction, was investigated. DAS and NSS were mixed in the ratio 40:1 at 4°C in HEPES buffer and left overnight to allow complete precipitation to occur. A suitable protocol for making a stock solution involved mixing 20 ml DAS, 0.485 ml NSS and 8.5 ml buffer. The fine precipitate formed was separated from the animal sera by centrifugation at low speed (1,000 g, 10 min) and decanting the supernatant. The precipitate was then re-suspended in the same volume of fresh buffer and stored at 4°C. The binding of primary antibody-tracer complexes with this reagent was significantly improved by this resuspension step and by washing the stock reagent several times with buffer, as described below.

To separate the bound and free fractions in the RIA incubates (total volume 330 µl), the stock DAS-NSS precipitate was diluted three-fold in buffer and, with continuous stirring, 40 µl was dispensed per tube. In contrast to the short second incubations with separating antibody covalently linked to solid-phase (e.g. SacCel), the use of pre-precipitated DAS-NSS required an overnight

incubation at 4°C. A HEPES buffer wash (1 ml) was added to tubes prior to centrifugation (30 min 2000 g, 4°C).

The improvement in the RIA achieved by washing the DAS-NSS stock reagent with buffer prior to use in the separation of an anti-T_4 antibody dilution curve, is shown in Figure 3.1. This caused a shift of the curve to the right, and a greater displacement of antibody-bound tracer by 50 pmol/l of added antigen was obtained. Use of charcoal-treated DAS and NSS to remove endogenous animal thyroid hormones from the initial preparation, did not further improve the sensitivity potential of this separation system, Figure 3.1.

3.2.6 Pre-precipitation of the Primary Antiserum

In this separation system, a pre-formed immune precipitate was prepared as described in Section 3.2.5 but with the inclusion of the RAST primary antiserum in the reagent. To prepare the reagent DAS, NSS and anti-T_4 antibody (pre-diluted x 100) were mixed in the ratio 40:1:0.002. This stock reagent was stored in HEPES buffer at three times the concentration used at the separation stage and it was stable at 4°C for at least 3 months. The calculated dilutions of components in this stock reagent, the working reagent and the RIA incubate are given in Table 3.2. Omission of primary antibody gave the reagent used to assess NSB values. The RIA incubates

Table 3.2 Preparation and Component Dilutions of the Pre-precipitated Anti-T_4 Reagent

Reagent Component	Volume (ml)	Dilution		
		Stock Reagent	Working Reagent	RIA Incubate
DAS	20.000	1:1.45	1:4.35	1:36
NSS	0.485	1:60	1:180	1:1,450
*Anti-T_4	0.045	1:64,600	1:194,000	1:1,600,000
Buffer	8.500			
Total	29.000			

* x 100 dilution of neat RAST antiserum.

consisted of 250 µl standard, 40 µl tracer and 40 µl of anti-T_4 pre-precipitate (i.e. final volume 330 µl). This assay system appeared to be superior to the methods described previously.

Washing the stock precipitate with HEPES buffer improved the antibody binding of tracer as shown by the increase in B_o from 14% to 47% of the dose-response curves in Figure 3.2(A). The main effect was seen after only one wash with relatively minor improvements in the assay occurring with subsequent washes. The use of charcoal-treated DAS and NSS to prepare the pre-precipitate improved the characteristics of the assay still further with a B_o of 58% being achieved when this reagent was washed and re-suspended in fresh buffer (Figure 3.2(A)). Anti-T_4 dilution curves comparing the use of charcoal-treated and untreated DAS and NSS to prepare washed pre-

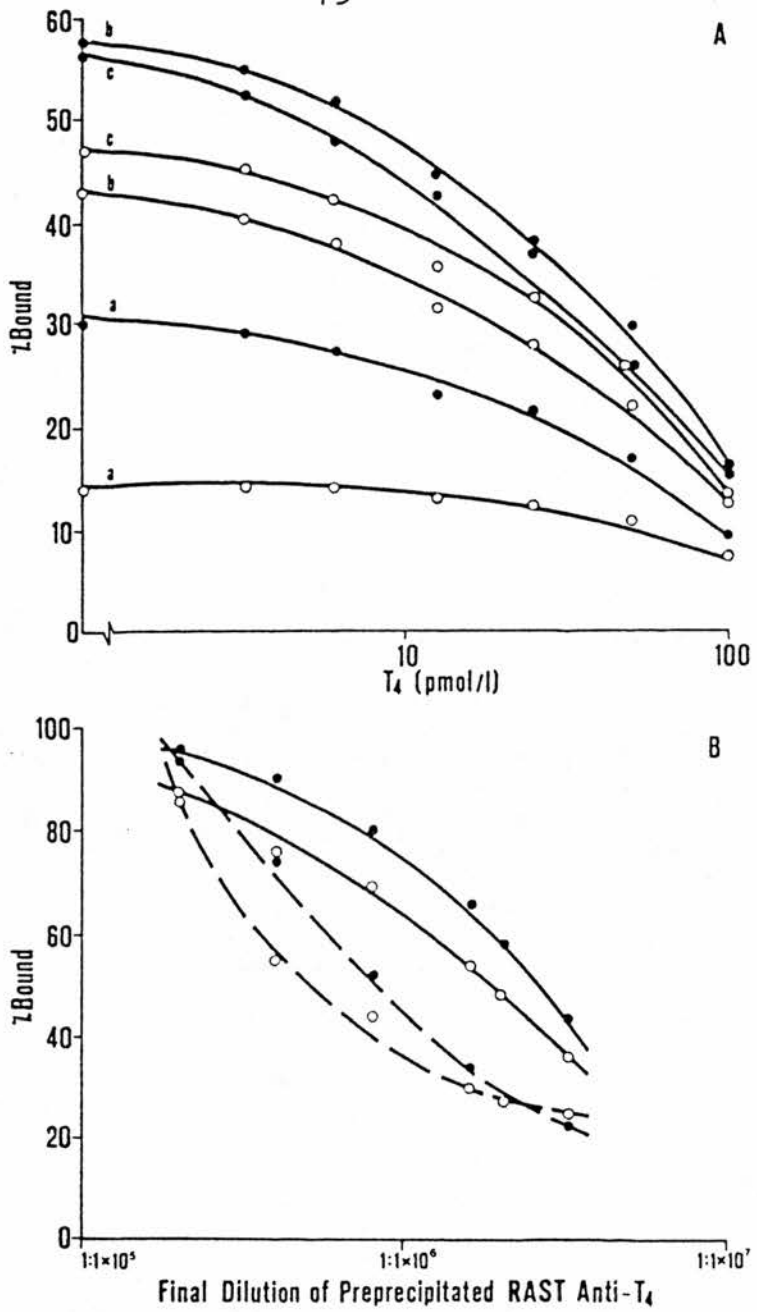


Figure 3.2(A) Standard Curves for T_4 Using Different Preparations of Pre-precipitated Anti-T_4 Antibody.

These were charcoal-treated DAS and NSS (●) or untreated DAS and NSS (o) which were either unwashed (a), washed x 1 (b) and washed x 3 (c) with fresh buffer.

(B) RAST Anti-T_4 Dilution Curves

The pre-precipitate was washed and made with either charcoal-treated (●) or untreated (o) DAS and NSS.
(zero antigen ——; 50 pmol/l T_4 ---).

precipitates (Figure 3.2(B)), confirmed the greater sensitivity potential of the former preparation.

As with other assays described using pre-precipitated double-antibody separation systems (Wong et al., 1979; Giles, 1982; Gray et al., 1983), standard curves for T_4 with good sensitivity were produced using short incubations at 37°C. The effects of altering the incubation conditions on the anti-T_4 dilution curve are shown in Figure 3.3. Incubation at 37°c for 3½-4 h was used in subsequent assays.

3.2.7 Comparison of Separation Methods

The main features of the different separation systems studied are summarised in Table 3.3. In the conventional post-precipitation double-antibody technique, interference at the primary antigen-antibody reaction produced low binding making it an unsuitable separation system for the high sensitivity T_4 RIA. It is likely that the presence of animal T_4 and TBG in the double-antibody reagents caused this interference by reducing the binding of ^{125}I-T_4 to the primary antibody. The use of a DAS-NSS precipitate resuspended in buffer removed this interference (Figure 3.1) whereas, in the use of pre-precipitated primary antibody, the T_4 binding sites would be exposed to animal T_4 during preparation of the reagent; this explains the improvement found in this separation system using charcoal-treated DAS and NSS (Figure 3.2).

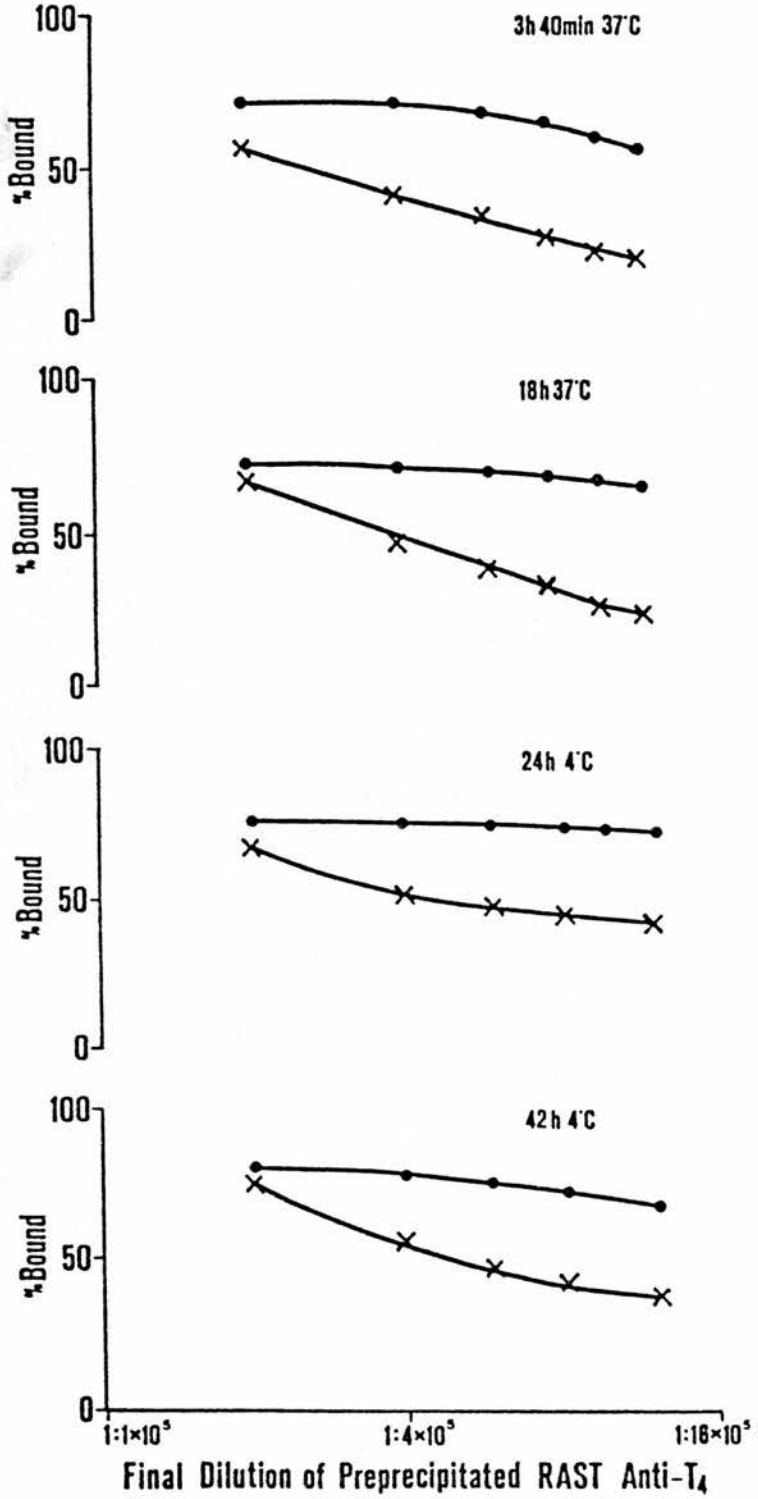


Figure 3.3 The Effect of Different Incubation Conditions on Dilution Curves of Pre-precipitated RAST Anti-T4 Antiserum.

Zero antigen (•—•), 50 pmol/l T4 (x—x).

Table 3.3 Comparison of Different Separation Systems in the RIA of Free T_4 in Serum Dialysates

Nature of Separation	Separation System				
	PEG precipitation	SacCel (cellulose-DAS)	Sephacryl-DAS	DAS-NSS Precipitate	Pre-precipitated Double-antibody
	Fractional precipitation of immunoglobulins	Solid-phase separating antibody (covalent)	Solid-phase separating antibody (covalent)	Solid-phase separating antibody (non-covalent)	Solid-phase primary antibody (non-covalent)
Primary antigen-antibody complex	Soluble	Soluble	Soluble	Soluble	Insoluble
Primary incubation	>3 h r.t.	>3 h r.t.	>3 h r.t.	>3 h r.t.	>3 h 40 min 37°C
Second incubation	15 min r.t.	30 min r.t.	1 h r.t.	>18 r.t.	-
No. of pipetting steps	5	4	4	4	3
Optimum RAST anti-T_4 final dilution	$1:2 \times 10^6$	$1:2 \times 10^6$	$1:2 \times 10^6$	$1:2 \times 10^6$	$1:1.6 \times 10^6$
No. of washes to give <5% NSB	2	2	2	1	1
Relative cost	+	+++	+	0	0

The use of the DAS-NSS precipitate as separating agent required a considerable incubation time. This may have been due to steric hindrance of the binding of primary antibody to unoccupied binding sites in the preformed DAS-NSS lattice. Thus, although this reagent was readily prepared and produced low NSB values, the long incubation time required was a disadvantage in view of the proposed coupling of the RIA to the overnight dialysis of serum.

The covalent solid-phase double-antibody methods were expensive and tended to have high NSB. In addition, frequent preparative chemistry was required to supply sufficient sephacryl-DAS for several assays.

The PEG precipitation system was simple and inexpensive, the disadvantages being the number of pipetting steps, the pipetting of the viscous PEG solution and the high NSB.

Use of the pre-precipitated double-antibody method reduced the number of incubations, pipetting steps and wash steps required to produce acceptable NSB values, making it the most convenient method to perform. The fine precipitate formed, probably accounts for the improved reaction kinetics observed with this system, compared to those found for primary antisera covalently linked to solid matrices e.g. micro-crystalline cellulose. The necessity to use charcoal-stripped DAS and NSS with this

system was not a major drawback since, after bulk preparation, these reagents, could be stored in appropriate quantities at -20°C until required. However, this method has been criticised in the past since a loss in sensitivity compared to separations of soluble primary antibody-antigen complexes may result (Hunter, 1973). This may be due to a decrease in the affinity of the primary antibody when pre-precipitated with the separating antibody. (This point is addressed in Section 3.3). It is of note that, whereas the chemical covalent coupling of primary antisera to solid-phase results in a substantial loss in antibody titre (Wide, 1969), there was little loss using the more gentle process of double-antibody pre-precipitation.

Since sensitivity is affected by many factors, this system was further investigated and developed in conjunction with the PEG precipitation method. The mean within-assay precision profiles from 10 assays are compared for these two separation systems in Figure 3.4, indicating a similar working range (CV <10%) for both methods. The lower profile in the case of the pre-precipitated double-antibody method indicated better within-assay precision compensating for any loss in sensitivity arising from changes in antibody affinity.

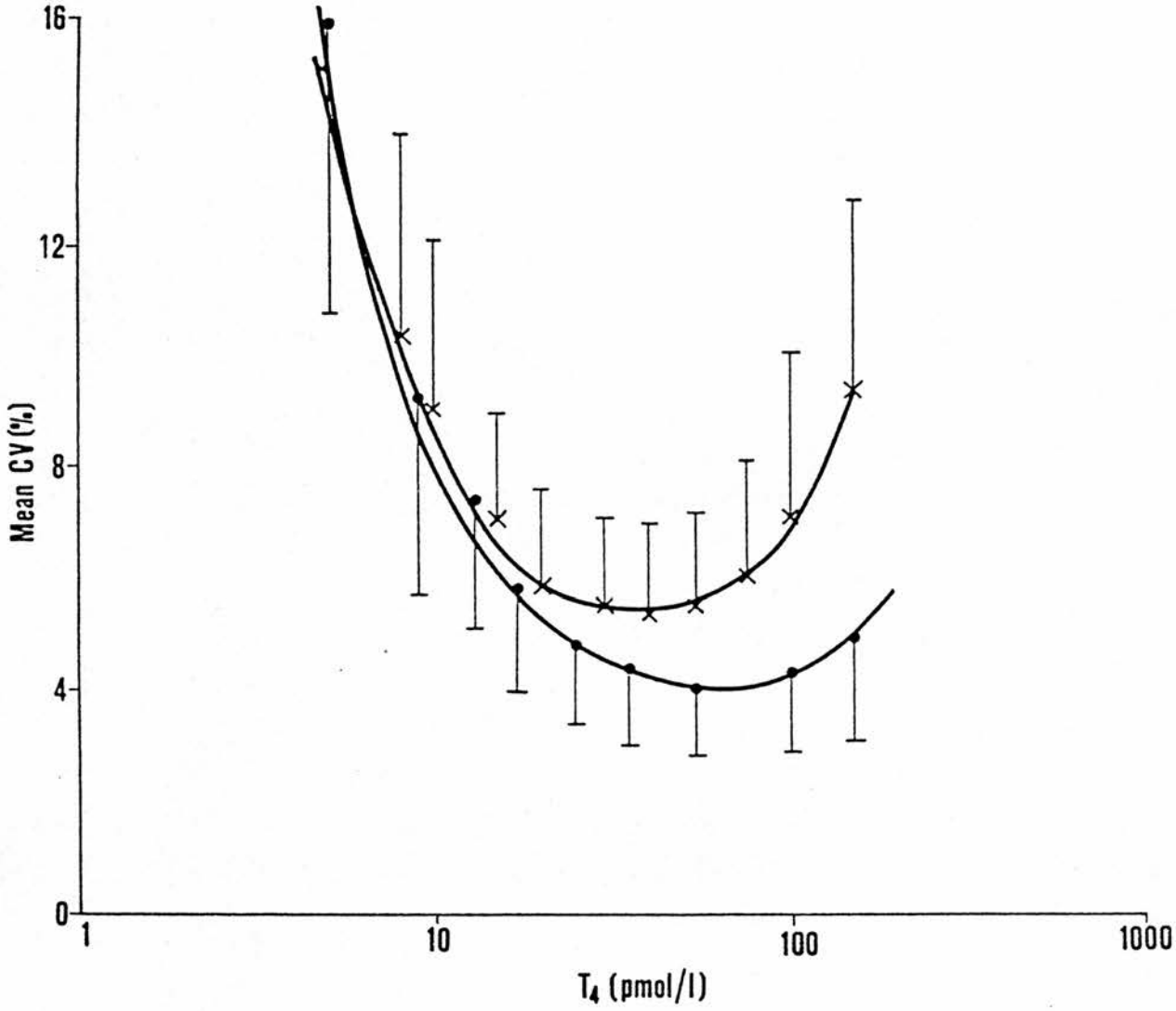


Figure 3.4 Within-assay Precision Profiles for the Sensitive RIA of T_4 (Mean ± SD, n = 10).

Separation was by PEG precipitation (x——x) and pre-precipitated RAST antibody (●——●).

The pre-precipitated double-antibody method was therefore chosen as the most simple, specific and suitable separation system for the RIA of free thyroid hormones in dialysates of serum. A similar method for the direct RIA of T_4 in serum dialysates has also been adopted by other workers (Giles, 1982).

3.3 OPTIMIZATION OF THE T_4 RIA FOR MAXIMUM SENSITIVITY

The theoretical sensitivity attainable in an RIA can be expressed as:

$$\frac{\text{experimental error in estimating the response}}{\text{affinity constant}}$$ (Ekins, 1974)

For maximum sensitivity it is desirable to select optimal reagents and conditions which reduce the error in the measurement of the bound radiolabel. This error combines those due to counting radioactive disintegrations, pipetting and the misclassification errors due to inefficiency of the separation system.

Since the error in counting radioactive disintegrations is approximated by $\sqrt{\text{counts}}$, this error can be reduced to <1% by accumulating >10,000 counts per tube. The combined counting and pipetting error (CV) found for the repeat dispensing of 40 µl of T_4 radiolabel by Microlab M in the sensitive T_4 RIA, was 1.8% when counts were >10,000.

Errors in pipetting can be reduced by increasing the volumes of solutions dispensed and by minimising the

number of pipetting steps performed. In the measurement of pmol/l concentrations of T_4 however, it is necessary to add antiserum and radiolabel to RIA incubates in as small a volume as possible to avoid excessive dilution of the analyte and loss in assay sensitivity.

Misclassification errors occurring at the separation stage of the RIA were least variable using the pre-precipitated double-antibody method (Section 3.2) which consistently gave NSB values less than 5%.

For very low concentrations of analyte, it becomes more important to use antisera with high avidity to achieve the required assay sensitivity. In the measurement of T_4 concentrations in dialysates of serum, primary antisera with affinity constants (K_a) $>10^{11}$ l/mol were thought to be suitable. In this thesis, four primary anti-T_4 antisera were investigated. Reagent volumes and incubations were then optimised to obtain the best precision profiles for the measurement of T_4 concentrations in the range 5-100 pmol/l.

3.3.1 Choice of Primary Anti-T_4 Antiserum

Primary antisera were assessed using incubations in 0.01M HEPES buffer pH 7.4 and ^{125}I-T_4 tracer. The K_a for each antiserum was calculated by transformation of typical dose response curves into a Scatchard plot (Scatchard *et al.*, 1949) and determining the initial slope

of the line obtained. The FD of antiserum required to bind 50% of added ^{125}I-T_4 (antibody titre) was determined by incubation of doubling dilutions of antiserum with ^{125}I-T_4 (3.5 fmol) in 300 μl of buffer at 4°C overnight. Separation was performed either by PEG precipitation (Section 3.2.2) or by using the antiserum pre-precipitated with DAS and NSS. The specificity details of the antisera are expressed as the percentage crossreaction with other iodothyronines by the method of Abraham, 1969.

Scatchard plots for the RAST antiserum used in soluble and pre-precipitated form are shown in Figure 3.5. The K_a values are shown in Table 3.4 with those calculated similarly for the 3 other antisera. The SAPU antiserum was approximately 10 times less avid than the 3 other antisera which had similar K_a values of the order of 4×10^{11} l/mol. Pre-precipitation of the antisera reduced K_a values by approximately 40%.

Table 3.4 Affinity Constants (K_a) of Primary Anti-T_4 Antisera and the Effect of Pre-precipitation

T_4 Antiserum	K_a (l/mol)	
	Soluble	Pre-precipitated
SAPU	4.1×10^{10}	2.8×10^{10}
RAST	4.5×10^{11}	2.3×10^{11}
ALS	4.0×10^{11}	-
Amersham	-	2.2×10^{11}

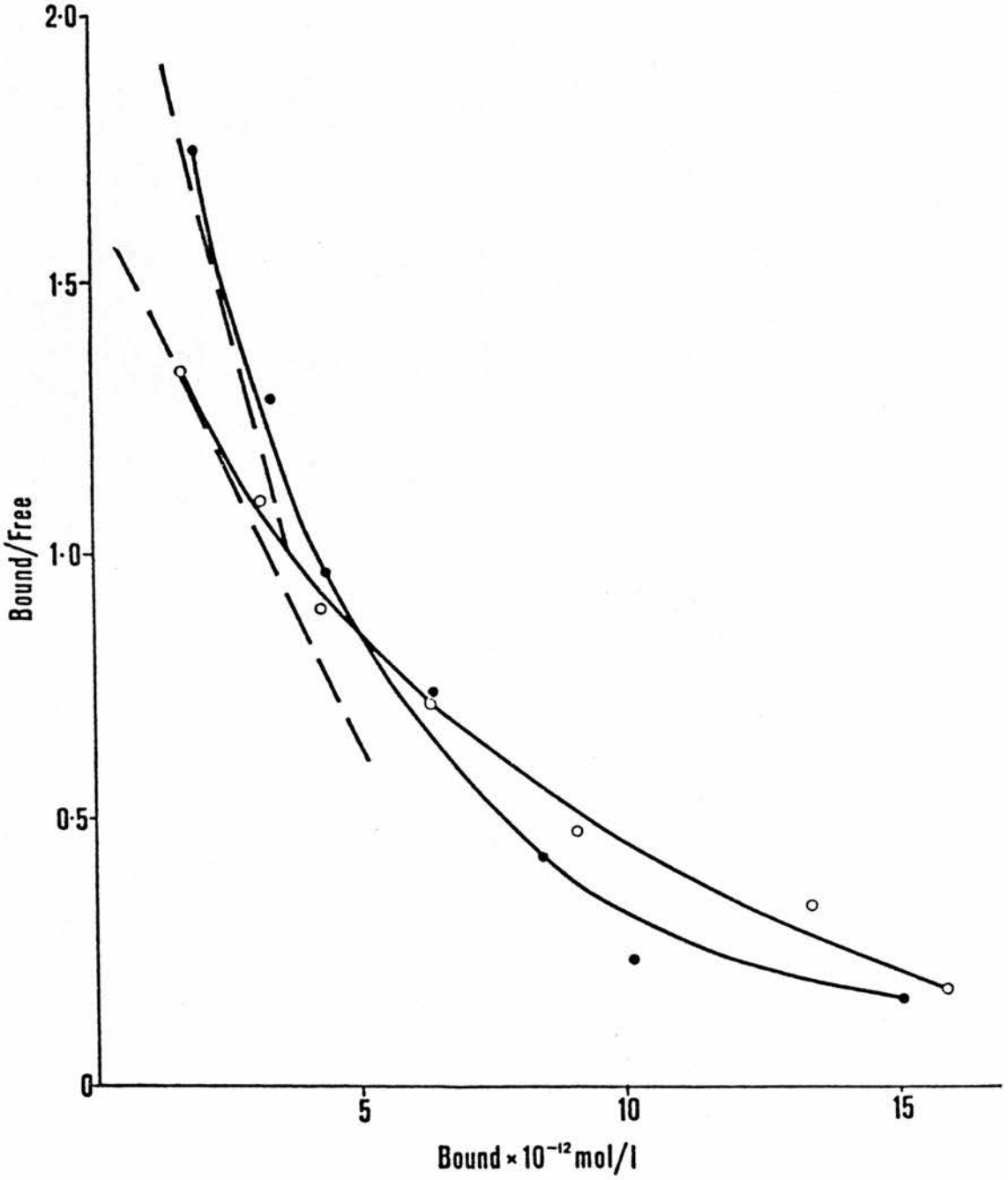


Figure 3.5 Scatchard Analysis of the RAST Anti-T4 Antiserum Used in Soluble (•) or Pre-precipitated Form (o).

The antibody titres using the antisera in the soluble or pre-precipitated form are shown in Table 3.5. Pre-precipitation resulted in a 20 - 30% reduction in titre. The ALS antiserum had lowest titre but the material obtained was at an undisclosed dilution of the original neat antiserum. Five to ten times the number of assays were possible with the RAST or Amersham antisera compared to that from ALS.

Table 3.5 Antibody Titres of Primary Anti-T_4 Antisera Used in Soluble or Pre-precipitated Form

T_4 Antiserum	Final Dilution binding 50% Label	
	Soluble	Pre-precipitated
SAPU	-	1:380,000
RAST	1:2,200,000	1:1,800,000
ALS	1:47,000	1:33,000
Amersham	-	1:3,300,000

The cross-reactivity data (Table 3.6) suggested sufficient specificity of all the antisera for the measurement of T_4 in dialysates of serum. From the literature, the ratio of T_4:T_3 in the dialysates of normal and hyperthyroid samples would be expected to be 3:1 and 2:1, respectively; the ratio of T_4:rT_3 in normal serum dialysates being approximately 12:1 and in patients with non-thyroidal illness, 3:1 (Yeo et al., 1977). The

maximum distortion of fT_4 results due to high fT_3 or free rT_3 concentrations with any of these antisera would therefore be <2% and <3%, respectively.

Table 3.6 Cross-reactivity of Anti-T_4 Antisera

T_4 Antiserum	T_3	rT_3
SAPU	2.4%	-
RAST	0.3%	0.6%
ALS	2.2%	-
Amersham	3.0%	10.4%

The Amersham antiserum was not available until the latter part of this study, the RAST antiserum was therefore chosen for further use due to its high K_a and titre. Although a decrease in sensitivity potential was demonstrated when primary antisera were pre-precipitated, overall effects on assay precision and hence the working range, were more favourable with this system (Section 3.2.7). The FD of antiserum which gave the best precision profiles over several assays using delayed tracer addition (see below) was 1:1,600,000.

3.3.2 Addition of Radiolabel

Traditionally, high specific activity (SA) tracers have been used to reduce radiolabel counting errors and the required counting time. However, sometimes relatively little improvement in assay precision and sensitivity is gained (Ekins, 1974 & 1983b).

Comparison of SAPU anti-T_4 dilution curves using addition of ^{125}I-T_4 SA >5590 μCi/μg (New England Nuclear Research Products, Stevenage, Herts, UK) in varying concentrations to produce a ten-fold difference in final concentration of radiolabel (i.e. 0.7-7.0 pmol/l), resulted in only a two-fold increase in the antibody titre. Reduction of the tracer mass could not, therefore, significantly improve the sensitivity limit of assays using this system.

Assays of adequate sensitivity (minimum detection limit 2 pmol/l) were achieved using the more readily available radiolabel from Amersham International (SA >1200 μCi/μg) with the more avid RAST or Amersham primary antisera. The final concentration of radiolabel in the incubates was kept initially at <5pmol/l (i.e. 3.5 fmol/tube, approx. 6000 cpm) and tubes were counted for 5 min. This tracer addition is similar to that described by others using tracers with SA >1000 μCi/μg (Jiang & Tue, 1977; Yeo et al., 1977a).

Sequential saturation analysis has been most effectively applied to the RIA of large molecules e.g. peptide hormones, where the binding of antigen to antibody is essentially irreversible. A two to four-fold improvement in assay sensitivity can result (Hunter, 1973). For small analytes re-equilibration occurs more quickly after the delayed addition of radiolabel and little improvement

in sensitivity results, unless the second incubation with radiolabel is short.

The effect of delaying the tracer addition in the sensitive RIA of T_4 using RAST pre-precipitated antiserum (FD 1:1,600,000) is shown in Figure 3.6(A). Standards were incubated either with antiserum and radiolabel for 3 h 40 min or with antiserum (3 h) followed by radiolabel (40 min). There was a reduction in B_o and NSB with the delayed addition of tracer and, assessed over several assays, the working range (CV <10%) was improved from 8-100 pmol/l to 5-100 pmol/l, by this procedure.

In practice, it was found that with this sequential addition of tracer, doubling the tracer mass added to 7 fmol/tube had no detrimental effect on the precision profiles obtained, which permitted shorter counting times. This was in agreement with Ekins's theoretical analysis (Ekins, 1974) and with the method of Giles (1982) who added 8-10 times the mass of ^{125}I-T_4 (25 fmol) compared to others (Ellis & Ekins, 1975; Jiang & Tue, 1977; Yeo et al., 1977a) who were using simultaneous antibody and tracer incubation.

3.3.3 Incubation Volumes

Combining the addition of antibody and tracer reagent in one step to reduce pipetting errors and minimise the dilution of dialysates resulted in poorer precision profiles than those achieved with separate and delayed tracer addition.

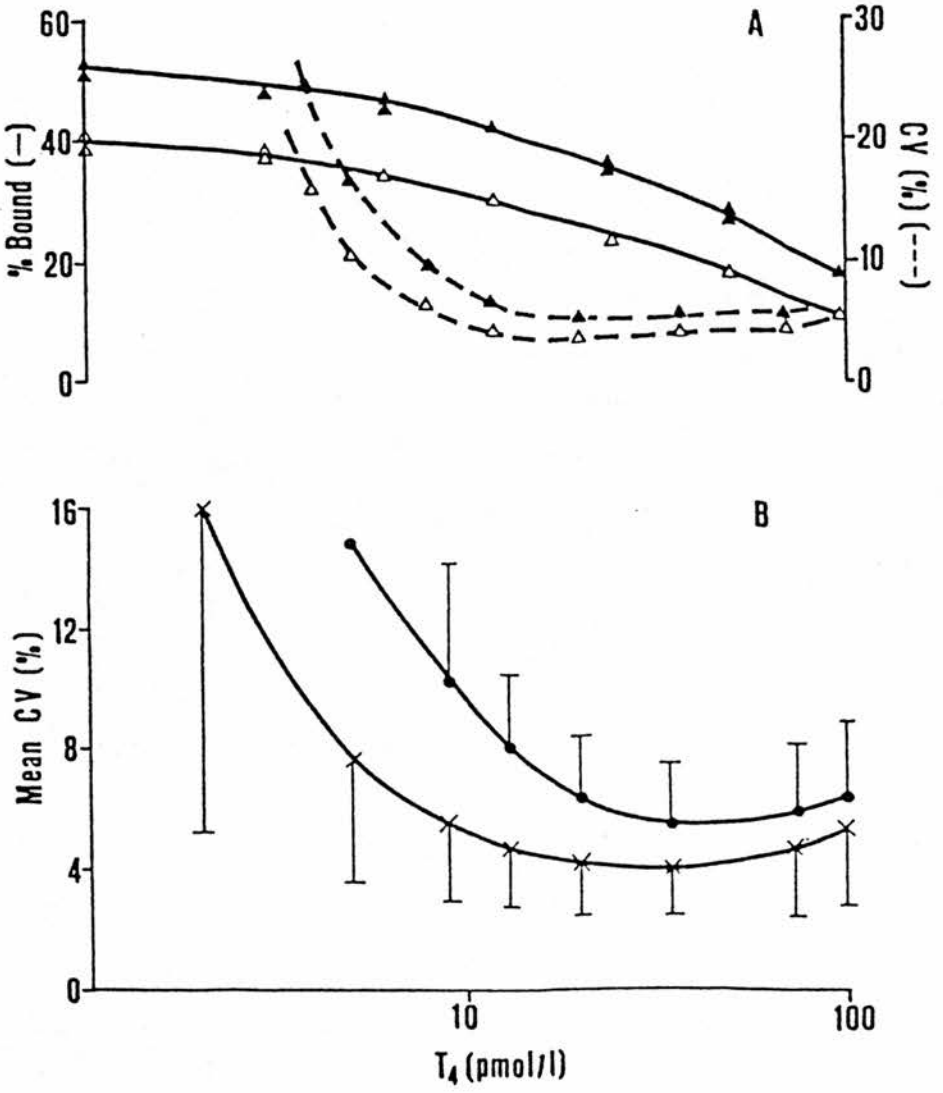


Figure 3.6 (A) The Effect of Sequential Tracer Addition on Standard Curves and Precision Profiles in the Sensitive RIA for T4.

Simultaneous (▲——▲) and sequential (Δ——Δ) tracer addition.

(B) The Improvement in the Within-assay Precision Profile (Mean ± SD, n = 10) Using a 500 µl (x—— x) Compared with 250 µl (•—— •) Standard Volume.

Mean within-assay precision profiles were compared for standards from 10 assays using delayed tracer addition (7 fmol/tube), pre-precipitated RAST antibody (FD 1:1,600,000) and (a) 250 µl standard + 20 µl antibody + 20 µl tracer or (b) 500 µl standard + 40 µl antibody + 40 µl radiolabel (Figure 3.6(B)). The larger volumes added resulted in an improvement in the precision profile and hence the sensitivity of the assays.

3.3.4 Procedures to Reduce Non-specific Binding

The presence of carrier protein (0.3% gelatine) in the RIA incubates was essential to prevent adsorption of tracer to the polystyrene tubes. The NSB of tracer to the antibody pellet was investigated using a pre-precipitate prepared omitting the primary antibody. Effects on the NSB of the delayed tracer addition and the use of buffer wash solutions containing different carrier proteins, are shown in Table 3.7.

Table 3.7 The Effect of Sequential Tracer Addition and Different Wash Solutions on NSB Values

Wash Solution*		NSB (%)	
		Tracer Addition	
Carrier Protein	No. of Washes	Simultaneous	Sequential
0.3% gelatin	1	8.8%	5.3%
	2	6.0%	3.9%
0.01% BSA	1	5.5%	3.0%
	2	3.4%	2.2%

* 0.01M HEPES buffer pH 7.4 containing 0.1% Brij.
BSA = Bovine serum albumin.

Inclusion of 0.01% BSA in the wash solution produced acceptable NSB values using only one wash prior to centrifugation in the sequential incubation of RIA components and, therefore, this was used in further assays.

3.3.5 Protocol for the Sensitive T_4 RIA

The proposed protocol for the measurement of T_4 in serum dialysates is shown in Figure 3.7. Details of the preparation of dilute standard solutions and the addition of carrier protein to standards and dialysates are described in Section 3.4. A four-parameter logistic model was used for curve-fitting and data interpolation. The sensitivity of this assay was calculated from 40 replicates of the B_o. The concentration of T_4 giving a signal 2.5 SD from zero was 2.0 pmol/l. The working range (CV <10%) for duplicate standards was 3.5 - 100 pmol/l (Figure 3.6(B)) making the assay suitable for the measurement of T_4 in dialysates of serum.

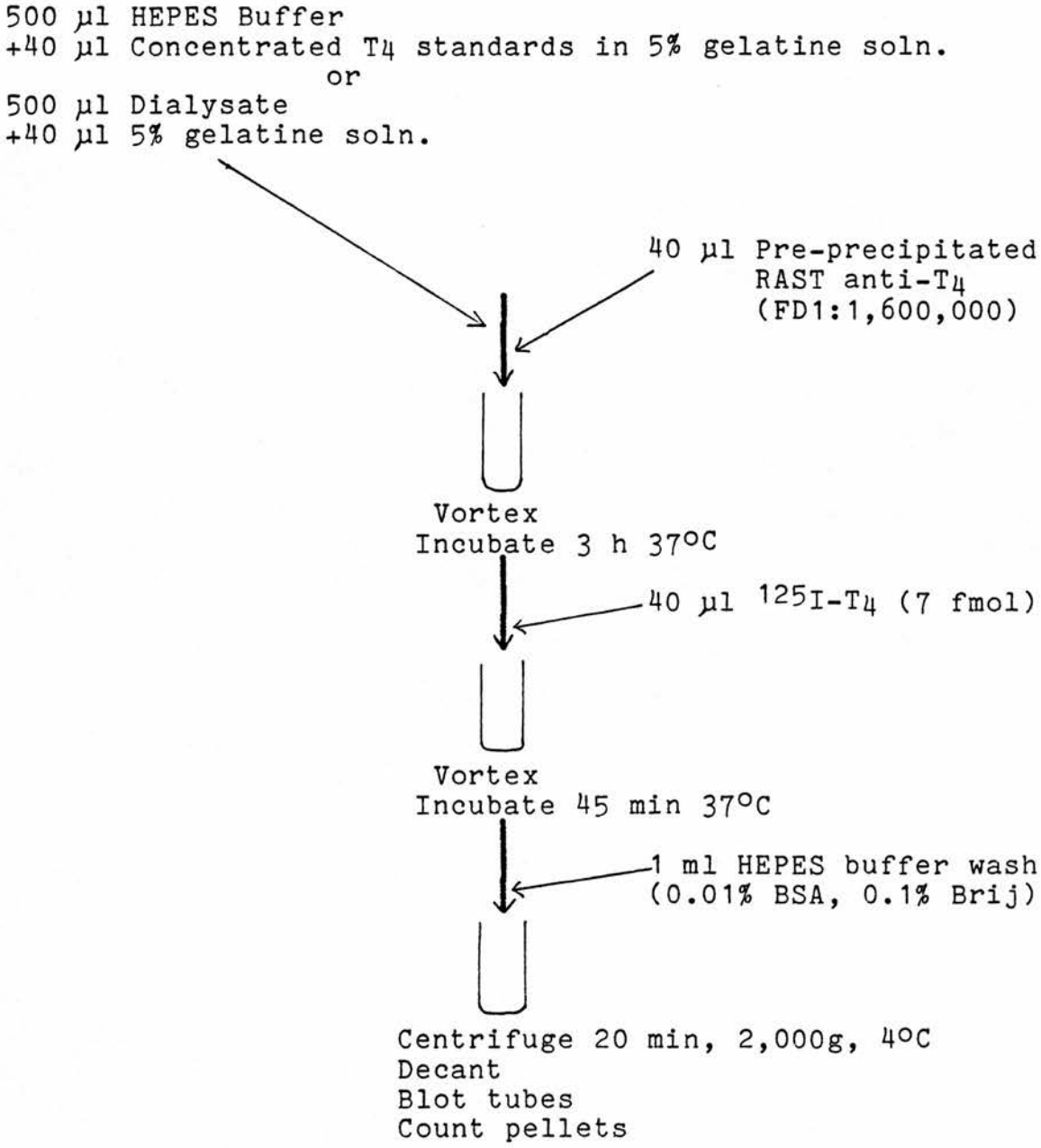


Figure 3.7 Final Protocol for the Direct RIA of T4 in Serum Dialysates.

3.4 PREPARATION AND STORAGE OF T_4 STANDARDS

The stock T_4 solution was prepared as described previously for the total T_4 assay (Section 2.2.1).

3.4.1 Preparation of Intermediate Standards

In order to avoid losses of T_4 due to adsorption onto glass or plastic, previous workers have used either silanized (neutral) glassware (Giles, 1982) or buffer containing carrier protein (Jiang & Tue, 1977; Yeo et al., 1977a) for dilutions of the stock standard. With inclusion of 0.2% (w/v) gelatine in the HEPES buffer, satisfactory (>95%) recovery of T_4 was demonstrated by monitoring added radiolabelled T_4 after each dilution stage, using untreated glass volumetric flasks and pipettes to make intermediate standards (10 µmol/l, 100 nmol/l, 1 nmol/l). A further check on the accuracy of these solutions was achieved by the analysis of dilutions made in T_4-free serum using the RIA for total T_4 (Section 2.2.2). These intermediate standards were used directly to make working standards for the sensitive T_4 RIA.

3.4.2 Preparation and Storage of Working Standards

Whereas other workers have diluted their stable stock solution to working standards prior to each assay, for ease of use and elimination of errors and contamination problems arising from making standards daily, it was decided to find a method of storing bulk quantities of working standards.

Initially, working standards, made by doubling dilutions (100, 50, 25, 12.5, 6.2, 3.1 pmol/l), were stored at -20°C in 4 ml aliquots in polystyrene RIA tubes. However, the standard curves generated using these large aliquots of standards had variable reproducibility and were dependent on the amount of carrier protein in the buffer (Figure 3.8) indicating significant losses by adsorption to the tube during storage. Two alternatives were tried: (a) working standards were stored at -20°C in RIA tubes at the correct volume which allowed the same tubes to be used for the RIA and (b) standards were stored in more concentrated form with a high concentration of carrier protein (5% gelatine).

(a) Frozen Working Standards

A large volume of each working standard in HEPES buffer containing 0.2% (w/v) gelatine was prepared and stored at -20°C in aliquots (500 µl) in RIA tubes. Any standard adsorbed to the plastic tube should be available to bind to the higher affinity binding sites of the added antibody. The standard curves produced were reproducible and, even after one month of storage, remained superimposable to standard curves made by diluting down from stock standard immediately prior to assay. When two T_4 pools in buffer were stored similarly to standards and interpolated from the standard curves in 8 assays, the between-assay precision (CV) was 14.1% and 7.8% at 12.0 and 23.5 pmol/l, respectively.

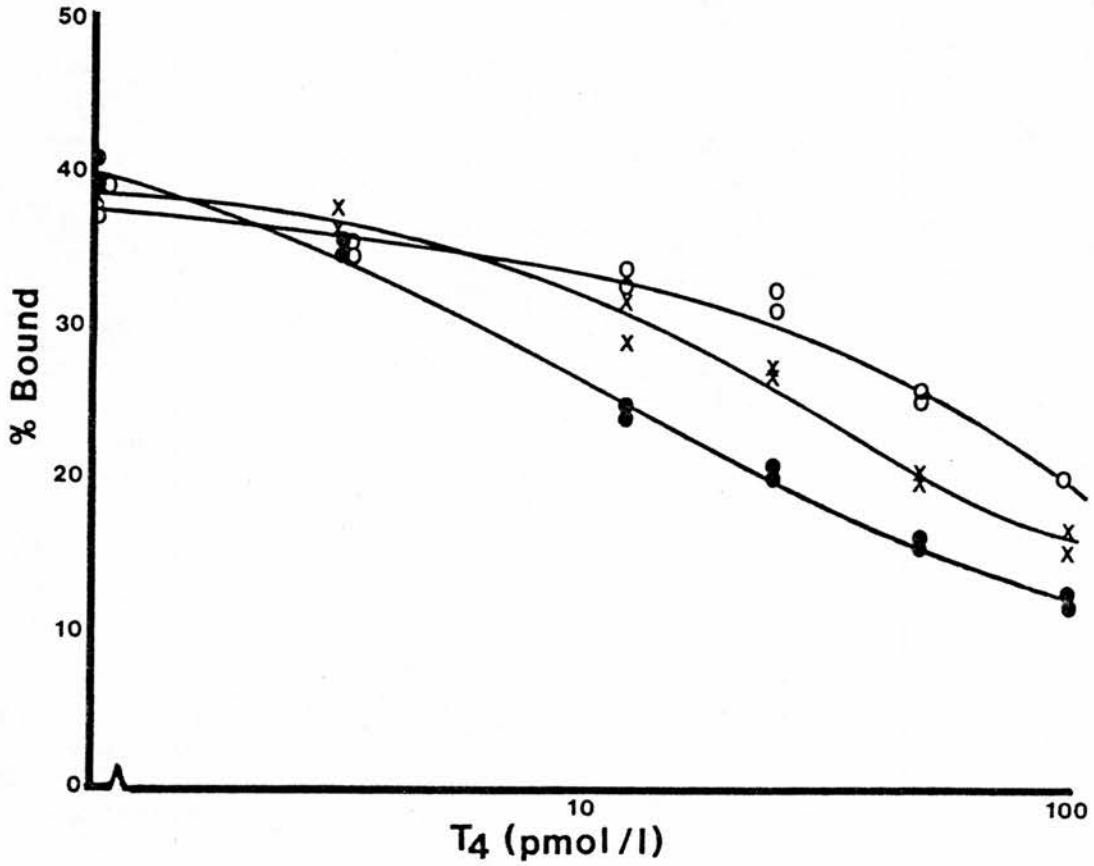


Figure 3.8 Free T_4 Standard Curves Using Standards Stored at -20°C Containing 0% (o), 0.2% (x) and 5% (•) Gelatine.

However, despite the potential of this method, the large number of tubes required for standard curves for 50-100 assays, made storage at -20°C impracticable.

(b) Storage of Standards at an Intermediate Dilution with a High Concentration of Carrier Protein

This method of storing standards had certain potential advantages:-

1. reduction in adsorption losses and instability of standards.
2. storage of standards for many assays.
3. addition of carrier protein to dialysates before RIA could be made similar to the procedure for pipetting the standard curve.

Since dialysates of serum would not contain carrier protein, the addition of gelatine in a small volume (and therefore high concentration) would be necessary at the RIA stage to reduce the NSB. Both Yeo et al. (1977a) and Giles (1982) added a small volume (50 µl) of gelatine solution in buffer (2% or 5% w/v) to their RIA incubates for this purpose. At 37°C the 5% gelatine solution can be pipetted reproducibly (Giles, 1982) but it solidifies at room temperature. The incorporation of carrier protein into dialysate and standard RIA incubates is shown in Figure 3.7 which represents the final protocol used in the clinical studies. The standards stored were 12.5 times more concentrated than those

described in (a) to allow for the dilution in the assay by diluent i.e. 1250, 625, 312, 156, 78 and 39 pmol/l. In solid gel form, these standards appeared equally stable stored at 4°C or -20°C but the latter was thought preferable for long time periods. Again this method produced standard curves which were very similar to those generated by dilution from the stock solution immediately prior to assay, even after several months and gave acceptable within-assay precision (Figure 3.9).

The between-assay precision (CV) assessed using three T_4 pools in buffer containing 5% gelatine (stored similarly to the standards) over a period of 2 months in 18 assays, was 13.1%, 12.8% and 10.1% at 7.7, 18.1 and 28.4 pmol/l, respectively. Further evidence for the stability of this method of standardisation is shown by the CUSUM plots for these pools over a period of 5 months with 3 changes of stock solutions used to make standards (Figure 3.10). Slight changes in bias for these pools tended to coincide with preparation of a new stock solution of T_4 and batch of standards. Further problems might have been encountered, therefore, had standards been diluted down from a stock solution prior to each assay.

3.4.3 The Choice of Diluent for Standards

Standards in T_4-free serum dialysate rather than buffer have been used by some workers to equalise any non-specific effects on antibody binding between dialysates of

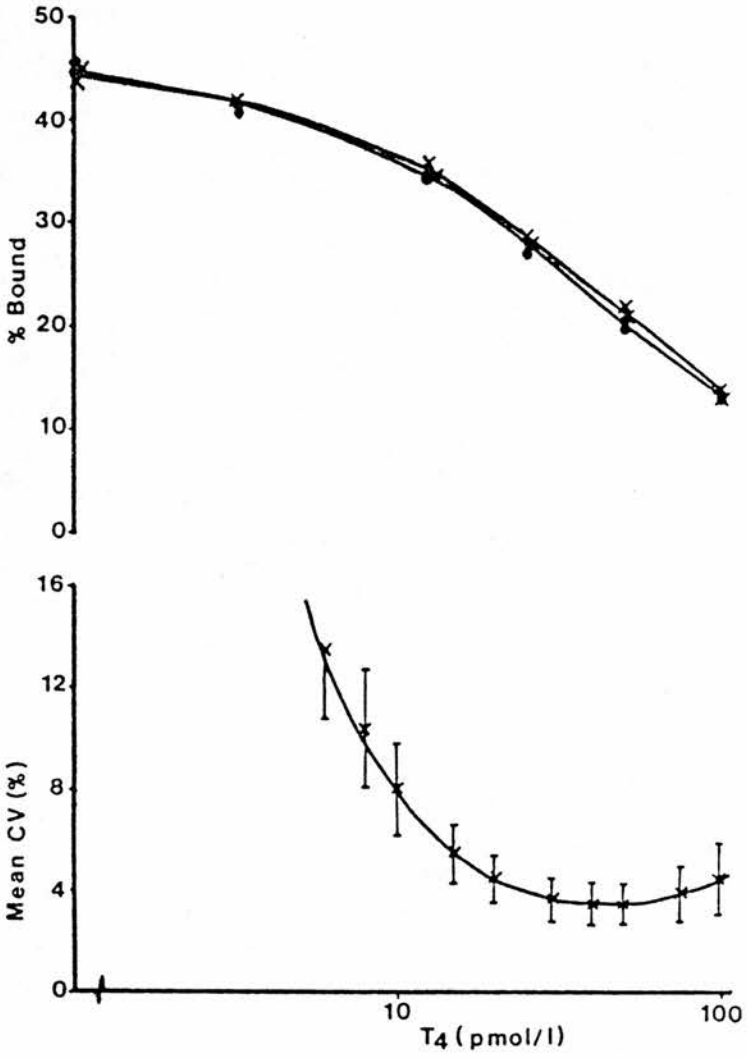


Figure 3.9 A: Comparison of T_4 Standard Curves

Standards freshly diluted from stock (•) and those stored concentrated in 5% gelatine solution (x) were compared.

B: A within-assay precision profile (Mean ± SD, n=8) of T_4 standards stored concentrated in 5% gelatine solution is shown below.

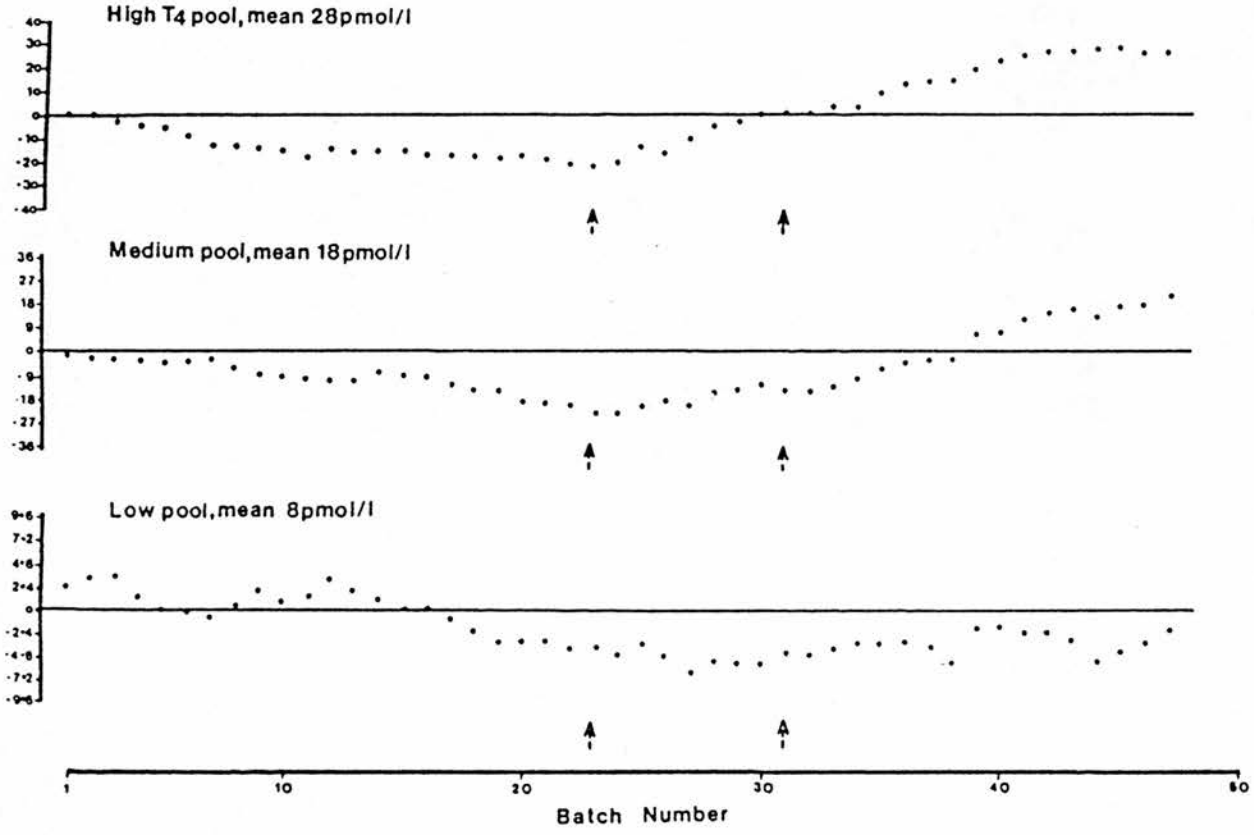


Figure 3.10 CUSUM Plots of Results for T4 Pools Stored in 5% Gelatine Solution Over Five Months.

Each division on the y-axis is equivalent to 2 SD from the mean T4 value.

Changes in the batch of standards with new stock solutions are indicated by an arrow.

serum and standards (Yeo et al., 1977a; Helenius & Liewendahl, 1983). However, others have found no detectable differences between the use of these two diluents Ellis & Ekins, 1975; Giles 1982).

In this RIA, the dialysate of charcoal-stripped human serum caused a slight displacement of tracer binding representing 1-3 pmol/l T_4. Standard curves using the two diluents showed a convergence of antibody binding for values greater than 12 pmol/l suggestive of residual T_4 being present in the dialysate rather than a non-specific matrix effect more likely to cause a constant displacement at all standard concentrations (Figure 3.11(A)). Pre-treatment of the dialysate with micro-crystalline-coupled anti-T_4 antiserum abolished the difference between buffer and dialysate curves supporting this explanation (Figure 3.11(B)).

Charcoal treatment of normal human serum removed 98.5% of T_4 (Section 2.2.1) leaving approximately 2 nmol/l of T_4 present. In addition, the bound-free equilibrium may be altered by charcoal treatment. It is unlikely, therefore, that the dialysate from this serum would be completely free of T_4, accounting for the 1-3 pmol/l displacement observed.

Dilution of four dialysates of serum three-fold with buffer also confirmed good linearity and absence of significant blank effects in the RIA (Table 3.8). It

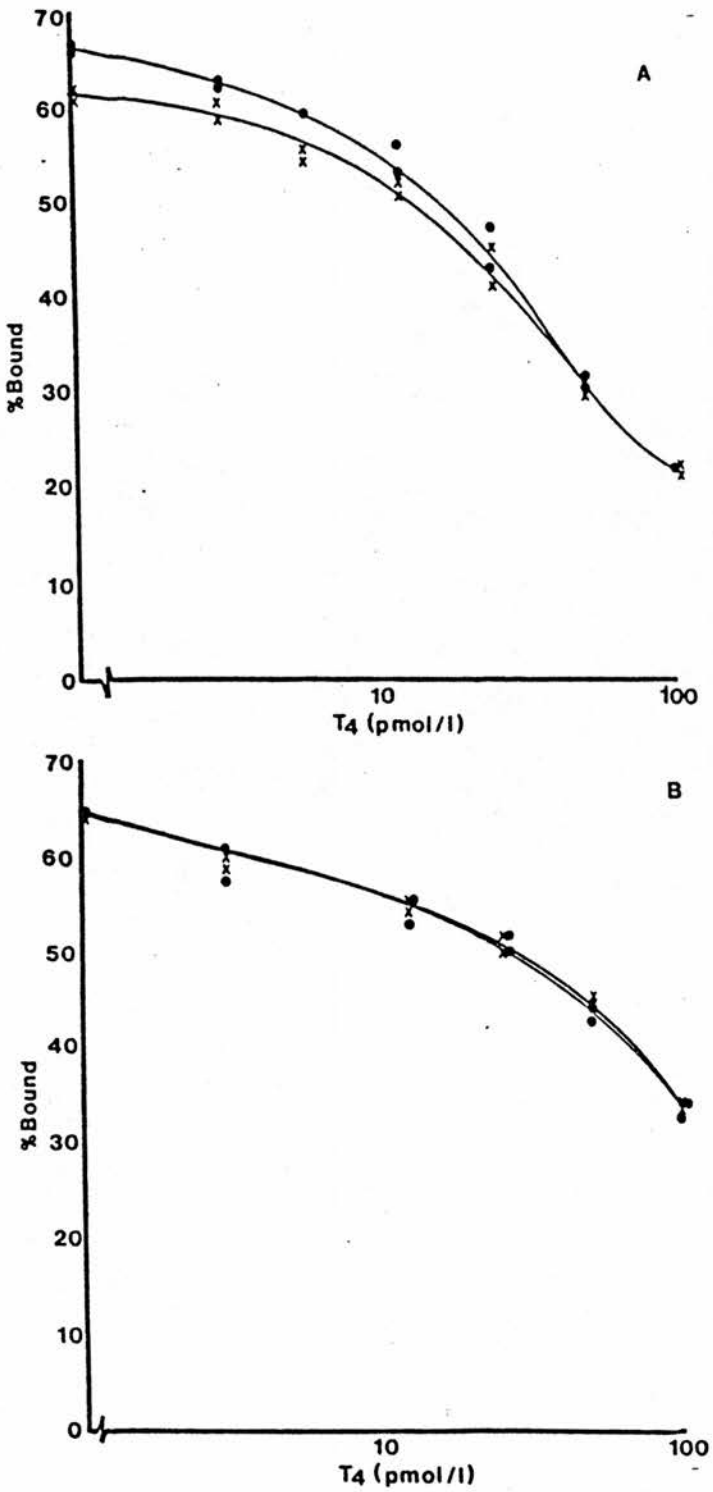


Figure 3.11 Comparison of T_4 Standard Curves.

A: Using buffer (•) and charcoal-stripped serum dialysate (x) as the diluent.

B: After pre-treatment of buffer (•) and charcoal-stripped serum dialysate (x) with solid-phase anti-T_4 antiserum.

was, therefore, decided to use buffer standard curves for the RIA of T_4 in dialysates of serum.

Table 3.8 Demonstration of the Lack of Blank Effects in the RIA of T_4 in Serum Dialysates

	T_4 (pmol/l)		
	Dialysate (undiluted)	Dialysate (x 3 diluted)	% Recovery
1.	42.7	14.7	103
2.	27.4	10.3	113
3.	24.5	8.0	98
4.	43.8	15.0	103

3.5 EQUILIBRIUM DIALYSIS

3.5.1 Dialysis Buffer

The buffer used for dialysis and pre-dilution of serum samples was 0.01 M HEPES containing 106 mmol/l sodium chloride and 1 mmol/l sodium azide. The pH at 37°C was adjusted to pH 7.4 by addition of sodium hydroxide. This buffer has been widely used for measuring free thyroid hormones (Ellis & Ekins, 1975; Yeo *et al.*, 1977a; Giles, 1982) but the inclusion of gentamicin as an additional bacteriostat has been recommended (Helenius & Liewendahl, 1983). The addition of gentamicin (15 mg/l) to the dialysis buffer did not affect antibody binding in the RIA nor the binding of T_4 to serum

proteins. (No significant difference was found for the fT_4 value of pooled normal serum dialysed in 10 dialysis cells using buffer ± gentamicin: fT_4 (-) 16.1 ± 0.9; fT_4 (+) 16.3 ± 1.3 pmol/l). It was therefore included in the dialysis buffer as a precaution against bacterial contamination.

Inclusion of 0.2% (w/v) gelatine in the dialysis buffer had no significant effect on the measured fT_4 in 10 paired samples dialysed against buffer ± gelatine. This suggested the absence of significant T_4-adsorption to the teflon cells. In accord with other methods described, carrier protein was therefore omitted in the dialysis step.

3.5.2 Preparation of Dialysis Membranes

Squares cut from Visking or Spectrapor seam-less tubing (7 x 7 cm) were opened out and soaked at 50°C in distilled water for 30 min, washed 5 times with tap water and 3 times with distilled water. Membranes were then stored at 4°C in dialysis buffer for up to 2 weeks. Washing the membranes in 10 mmol/l EDTA as recommended by Jiang and Tue (1977), was not necessary as interference due to factors deriving from the dialysis tubing was not evident in this system.

3.5.3 Dialysis Procedure

Membranes were clamped between the teflon half-cells of the Dianorm dialyser system (Figure 3.12).

Figure 3.12 The Equilibrium Dialysis Apparatus.

1. Teflon half-cells with semi-permeable membrane.
2. Rotator and rack assembly.
3. Microlab with sampling probe.

Four racks each consisting of 5 cells could be assembled on the rotator. Additions to and sampling from the half-cells was by means of entry ports which could be sealed with inert plastic stoppers. Diluted serum (1 ml) was added to one half-cell and dialysis buffer (1.1 ml) to the other. To aid sampling and the later washing of the teflon cells, serum was always placed in the male half-cell and buffer in the female half-cell. The dialysis was performed in a water-bath at 37°C ± 0.5 with rotation at 8 rpm. Dialysates were sampled by means of a cannula attached to the probe of a Microlab M diluter. The program sequence used to dispense dialysates directly into the tubes for RIA was:-

aspirate distilled water (200 µl)
sample air (50 µl)
sample dialysate (500 µl) } x 2
sample air (40 µl) } x 2
sample 5% gelatine at 37°C (40 µl) } x 2
dispense to RIA tube (600 µl) } x 2
aspirate distilled water (700 µl)
dispense total volume to waste

Each dialysate was analysed in duplicate for T_4 by RIA and 20 cells could be sampled in 30 min. To avoid contamination of the diluter with T_4, dialysates were always added to tubes for RIA before diluting the standards. After the addition of standards, the diluter tubing was washed with distilled water for 10 min before priming with the antibody reagent.

The teflon half-cells were cleaned by initial rinsing with tap water followed by overnight soaking with Pyroneg detergent. Half-cells were then rinsed several times with tap water and three times with distilled water.

3.5.4 Optimisation of Dialysis Time and Choice of Dialysis Membrane

A time-course of the dialysis using the Dianorm apparatus and the two different dialysis membranes is shown in Figure 3.13. Three patients' sera were pre-diluted 1:20 before addition to the dialysis half-cells. Plateau fT_4 values were reached after 16 h for both membranes. An overnight dialysis of 18-24 h was used in further assays.

There was no significant difference found for fT_4 values in 27 patients' samples using either Visking or Spectrapor membranes for dialysis: mean fT_4 (Visking) 13.4±1.7; mean fT_4 (Spectrapor) 14.1±2.6 pmol/l, $p = 0.09$. The Spectrapor membranes allowed slightly faster equilibration but this was of little practical advantage. The less expensive Visking tubing was therefore used for subsequent measurements.

3.5.5 Effects of Serum Dilution on Free T_4 Values

Free T_4 values showed little change when sera were diluted 2-80 fold before addition to the dialysis cell. Dilution profiles were performed on sera from hyperthyroid, euthyroid and hypothyroid patients and serum

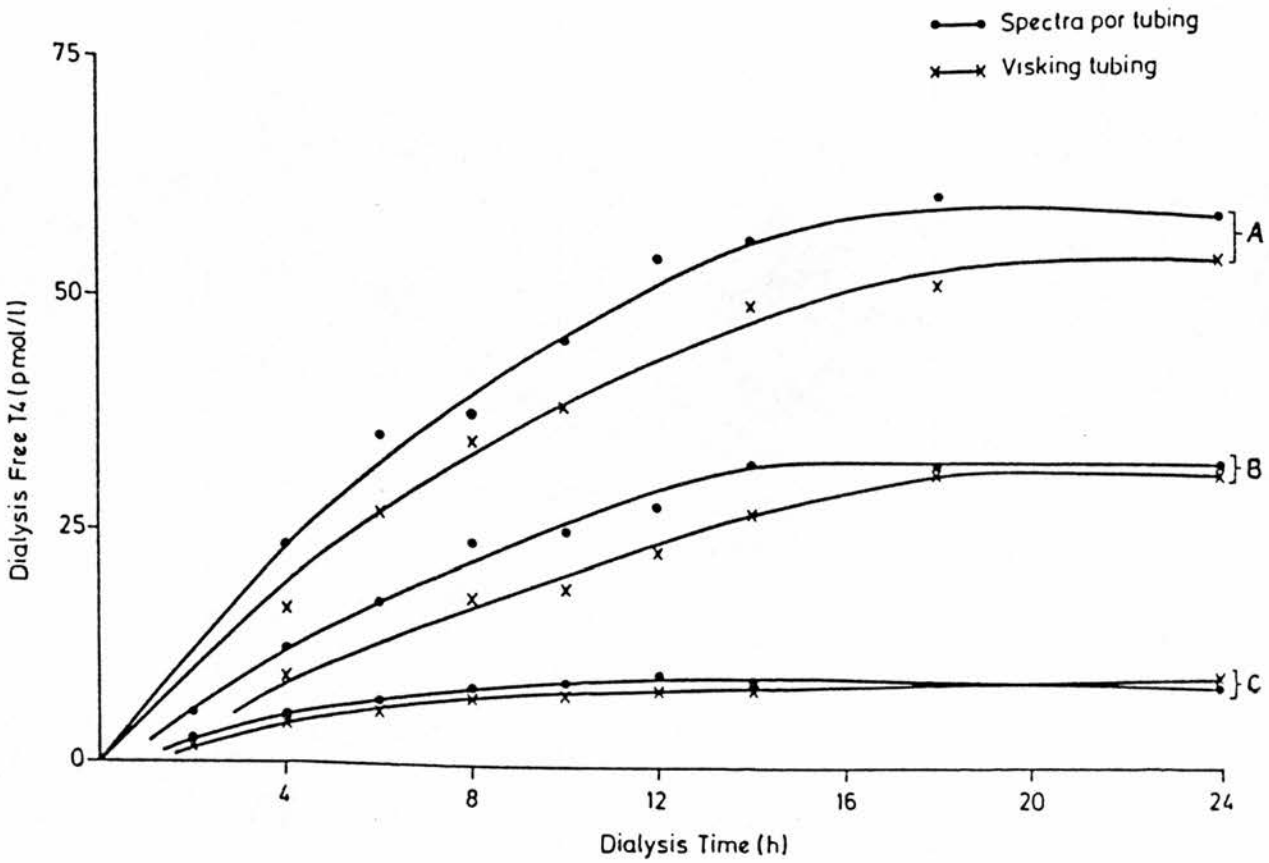


Figure 3.13 Time-course of Equilibrium Dialysis for Free T4.

Three patients' samples (A,B,C) were dialysed after 1:20 dilution.

pools containing added T_4 or charcoal-stripped serum (Figure 3.14). For further assays, serum was therefore diluted 1:20 with dialysis buffer prior to dialysis, reducing the requirement for serum to 50 µl per dialysis cell.

3.5.6 The Stability of Free T_4 in Samples at 4°C

Eight paired sera and plasma samples from patients attending a thyroid clinic were analysed for fT_4 on 4 days storing samples at 4°C. No significant difference in fT_4 values was found using serum or plasma comparing data within each dialysis run or using the mean values for each sample analysed on 4 days. Similarly there was no evidence of deterioration of measured fT_4 values in samples stored at 4°C for 4 days. Results for 6 paired samples which span a range of fT_4 values are shown in Figure 3.15. Slight changes in the values measured on different days could be attributed to between-assay variation assessed by the dialysis of freshly thawed serum pools on each day.

3.5.7 Within-assay Precision of the Free T_4 Dialysis-RIA

The mean within-assay precision profile for the RIA of dialysates of patients' sera (Figure 3.16) was very similar that for the analysis of standards (Figure 3.9). The working range of the RIA coupled to dialysis was 6.6-100 pmol/l fT_4.

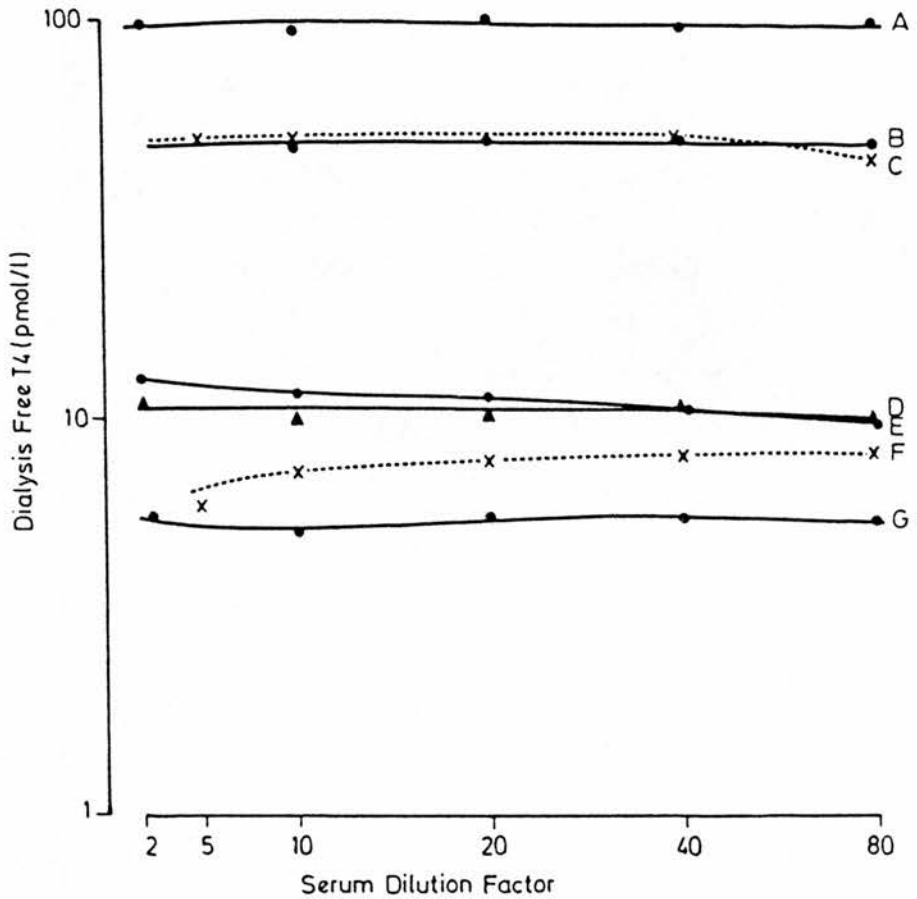


Figure 3.14 The Effects of Serum Dilution Prior to Dialysis-RIA of Free T_4.

Sera were from thyrotoxic patients (A,B), euthyroid patients (●——● E,▲——▲ D on OCP) and a hypothyroid patient (G). Two serum pools (x---x) with added T_4 (C) or diluted with charcoal-stripped serum (F) were also analysed.

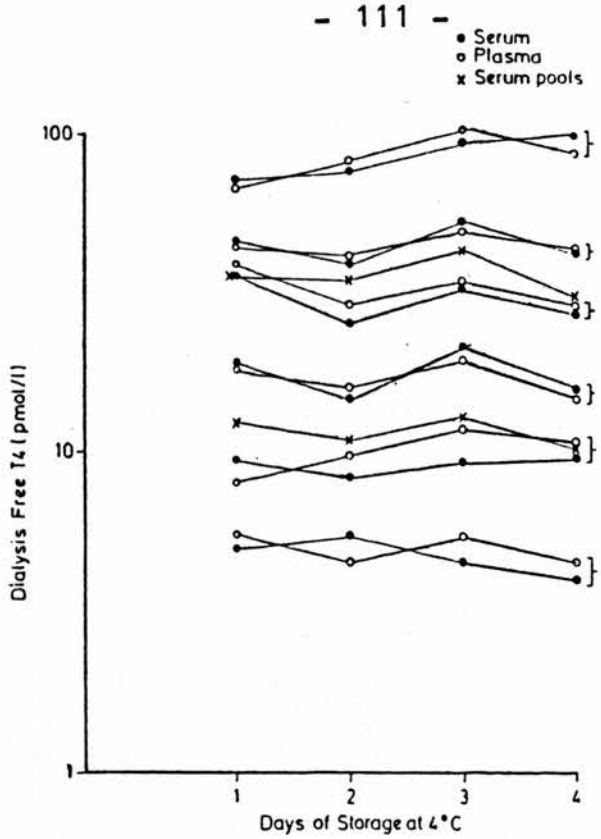


Figure 3.15 Dialysis Free T_4 Values in Paired Serum and Plasma Samples Stored at 4°C Over 4 Days.

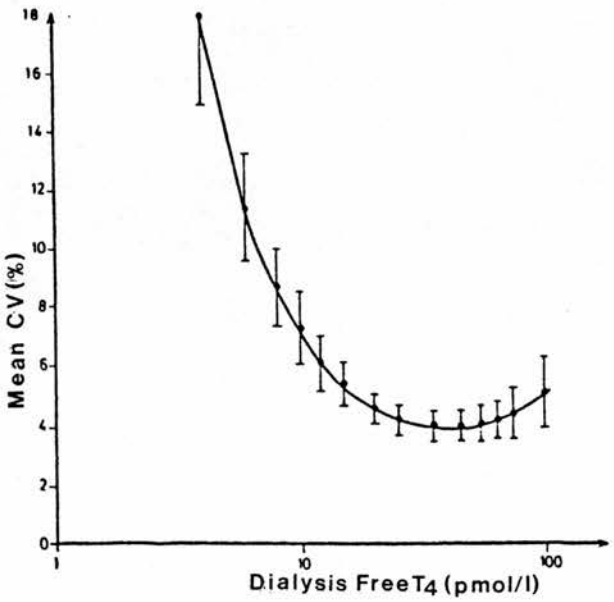


Figure 3.16 Within-assay Precision Profile (mean ± SD, n=30) for T_4 Measured in Dialysates of Patients' Sera.

The precision between cells in the same dialysis-RIA was assessed (a) by the dialysis of pooled serum in several cells and (b) by analysis of the variation in fT_4 values for patient samples dialysed in duplicate cells. The within-dialysis run CVs for 3 serum pools are shown in Table 3.9; the precision using the RIA with half volumes was also compared for two of the pools.

Table 3.9 Within-assay Precision of the Free T_4 Dialysis-RIA

Pool	n	Mean (pmol/l)	CV (%)	
			500 µl*	250 µl*
1	10	9.9	8.7	11.3
2	20	16.0	5.7	-
3	10	27.5	6.4	10.2

*dialysate volume

The poorer reproducibility using 250 µl of dialysate compared to 500 µl, precluded the dual measurement of both fT_4 and fT_3 in the dialysate from one cell. The variation in fT_4 results between two cells (RIA full volumes, 500 µl sample) was calculated for patient samples, Table 3.10. This analysis showed very good reproducibility only at high fT_4 concentrations therefore two dialysis cells per serum sample were used for subsequent fT_4 measurements, taking the mean result for the two cells.

Table 3.10 Within-assay Precision for Duplicate Dialysis Cells

	FT_4 range (pmol/l)		
	2 - 9.5	7.1 - 15.9	22.4 - 117.5
n	16	31	14
x_1 (pmol/l)	6.1	11.7	54.3
x_2 (pmol/l)	6.1	12.8	55.1
SD (pmol/l)	0.72	1.50	3.70
t	0.07	- 2.9	- 0.6
CV (%)	11.8	12.5	6.9

3.5.8 Between-assay Precision of the Free T_4 Dialysis-RIA

The between-assay precision of the fT_4 measurement was calculated in 2 ways (a) by the dialysis and RIA of quality control serum pools (in duplicate) for several dialysis runs and (b) by the repeat analysis of patients' sera between dialysis runs (RAC).

Outdated human serum pooled from blood transfusion service donors was used as the base material for dialysis-RIA pools. This pooled serum had a total T_4 concentration of 110 nmol/l and constituted the medium level pool. A low fT_4 level pool was prepared by mixing medium pool with charcoal-treated serum 1:1 by volume and a high fT_4 pool was prepared by adding T_4 standard to the base material to give a total T_4 concentration of 160 nmol/l. These pools were stable for at least six

months stored at -20°C in 0.2 ml aliquots and the precision (CV) calculated from their analysis in 20 dialysis-RIA runs is shown in Table 3.11.

Table 3.11 Dialysis-RIA Free T_4 Between-assay Precision (Serum Pools)

Pool	*Mean (pmol/l)	SD (pmol/l)	CV (%)
Low	7.2	0.85	11.9
Medium	13.0	1.98	15.3
High	27.8	2.73	9.8

*n = 20

The precision (CV) for RAC sera is shown in Table 3.12 for two ranges of values.

Table 3.12 Dialysis-RIA Free T_4 Between-assay Precision (RAC Sera)

n	Range (pmol/l)	Mean (pmol/l)		SD (pmol/l)	t	CV (%)
		1	2			
68	5 - 30	13.8	13.6	1.6	0.63	11.9
25	29 -106	49.5	49.8	3.7	-0.24	7.5

This reference fT4 method, therefore, had acceptable between-assay precision and CUSUM plots for the quality control sera over a period of 3 months with 3 changes of standards confirmed the reproducibility of the dialysis and RIA steps achieved over a prolonged time period (Figure 3.17).

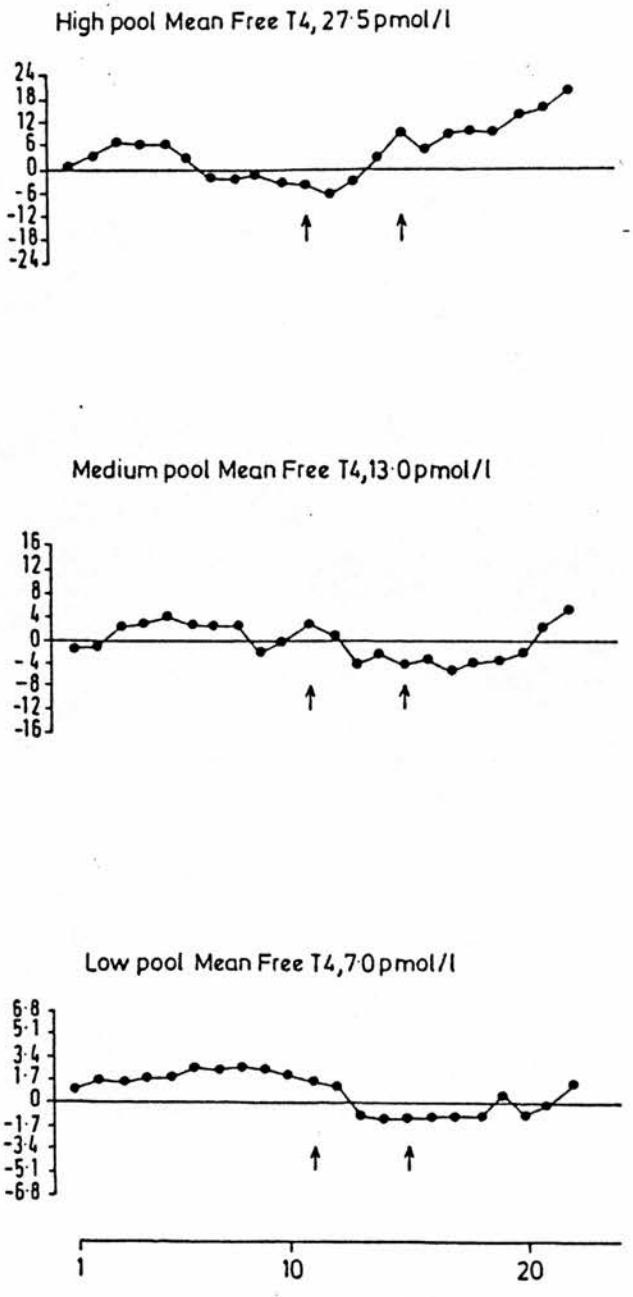


Figure 3.17 The Variability of the Free T_4 Values by Dialysis-RIA for Quality Control Sera.

The data are plotted as CUSUMS with the batch No. on the x-axis and each division on the y-axis equivalent to 2 SD from the target value for fT_4 (pmol/l).

3.5.9 Validation of the Reference Free T_4 Method

Serum samples collected from 162 patients attending a thyroid clinic were measured for fT_4 by the dialysis-RIA method (these were the first samples collected in a large series; a full description of these patients and criteria used to categorise them are given in Section 4.1). Samples from patients in different diagnostic categories were dispersed in separate dialysis runs. A clear separation between overt thyroid disease and euthyroidism was obtained for most patients (Figure 3.18). One 66 year old lady with Graves' disease who had no TSH response to TRH and who had total T_4 and T_3 results of 164 and 2.9 nmol/l respectively, had a normal fT_4 concentration. A fT_4 result within reference limits was also obtained using an Amerlex fT_4 kit although fT_3 (Amerlex) was at the upper limit of normal in this sample. There was a greater degree of overlap at the euthyroid-hypothyroid border which could be attributed to the poorer precision of the fT_4 dialysis-RIA at lower values. Some of the patients with subclinical thyroid disease had abnormal fT_4 values indicating the potential for greater discrimination of mild degrees of thyroid dysfunction using a measurement of free rather than total thyroid hormone concentrations.

The fT_4 results from the 63 patients classified clinically and biochemically as euthyroid, had a normal

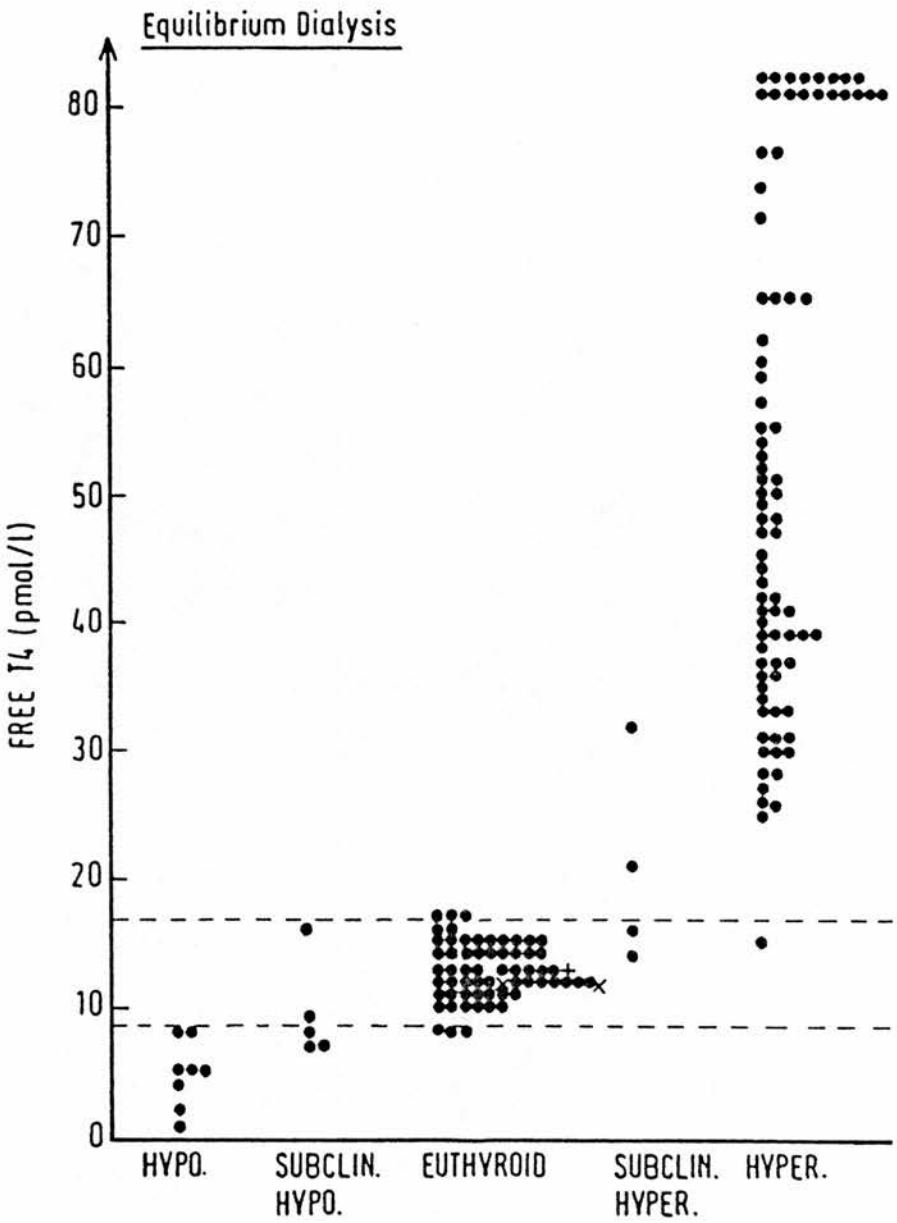


Figure 3.18 Free T4 Concentrations by Dialysis-RIA in 162 Patients from a Thyroid Clinic.

Two euthyroid patients were taking an OCP (x) and one had high-affinity binding of T4 to albumin (+).

distribution and these were used to derive a reference interval (mean ± 2SD) of 8-17 pmol/l for the method. Three euthyroid patients had altered hormone-binding proteins in their serum; two had high TBG levels induced by oestrogen and one, described later in more detail (Section 5.2.2), had increased binding of T_4 to variant albumin. The reference method was clearly unaffected by altered distribution of total hormone binding in serum.

3.5.10 Comparison of Direct Equilibrium Methods for Free T_4

Other direct methods for the measurement of fT_4 in serum were described during and after the development of this dialysis-RIA method (Table 3.13). In general, these and earlier direct methods (Table 1.3) have similar reproducibility, sensitivity and reference values as the present method. These other studies have also confirmed the absence of significant dilution effects on fT_4 values for overall dilutions of serum in the dialysis cell of 1:4 - 1:80. Nelson & Weiss (1985) have also reported little increase in values when neat serum is dialysed against the same buffer volume (overall dilution 1:2) for euthyroid and hyperthyroid fT_4 values <100 pmol/l; this does not, therefore, account for the higher reference values found using their method (Table 3.13). Slight differences in the ionic composition of the HEPES buffer used to maintain the dialysis of neat serum at pH 7.4 may be partly responsible.

Table 3.13 Comparison of Recently Described Direct Free T_4 Reference Methods

Free T_4

Reference	Method	Overall dilution of serum*	RIA separation	Precision (CV) Intra-assay	Precision (CV) Inter-assay	Sensitivity (pmol/l)	Ref.Range (pmol/l)
Giles, 1982	Equilibrium dialysis + RIA	1:40	Pre-precipitated double-antibody	-	13%	3	11-23
Helenius & Liewendahl, 1983	Equilibrium dialysis + RIA	1:54	PEG precipitation	5%	8%	2-3	9-21
This study	Equilibrium dialysis + RIA	1:40	Pre-precipitated double-antibody	6%	11%	2	8-17
Nelson & Weiss, 1985	Equilibrium dialysis + RIA	1:2	PEG precipitation	5%	–	2	11-31
Weeke et al., 1986	Ultrafiltration + RIA	1:1	PEG precipitation	11%	9%	–	14-47

*Dilution in both the serum and buffer compartments.

Weeke et al. (1986) have developed an ultrafiltration-RIA method for fT_4 which should avoid any perturbation of the fT_4 equilibrium due to serum dilution and altered ionic composition. However, this method is less precise and more susceptible to temperature changes and leakage of protein-bound hormone through the ultrafilter. These factors may account for the higher reference values they obtained (Table 3.13).

3.6 FREE T_3 MEASUREMENT BY DIALYSIS-RIA

The main interest in the measurement of fT_3 levels in serum is in distinguishing hyperthyroid from euthyroid states and, therefore, optimum assay precision is required at the upper limit of normal. Previous estimates by other workers using direct dialysis suggested this to be between 10 and 15 pmol/l (Ellis & Ekins, 1975; Yeo et al., 1977a; Weeke & Orskov, 1979). The assay was, therefore, designed to have optimal precision between 5 and 20 pmol/l.

3.6.1 Optimisation of the Sensitive T_3-RIA Procedure

The procedures developed for the sensitive T_4 RIA e.g. the use of pre-precipitated primary antiserum, the storage of standards in buffer containing 5% gelatine and the incubation conditions were all successfully applied to the assay of picomolar T_3 concentrations. The final protocol is shown below with a typical method for preparing the pre-precipitated antiserum (Table 3.14).

Protocol for the Direct RIA of T_3 in Serum Dialysates:

500 µl Dialysate
40 µl 5% gelatine in HEPES buffer

OR

500 µl HEPES Buffer
40 µl T_3 standard in 5% gelatine

↓

40 µl Pre-precipitated anti-T_3 (FD 1:2,300,000)

↓ Incubate 37°C 3 h

↓ 40 µl ^{125}I-T_3 (3 fmol)

↓ Incubate 45 min 37°C

↓ 1 ml HEPES buffer wash (0.01% BSA, 0.1% Brij)

↓ Centrifuge at 4°C 2,000 g for 20 min

↓ Decant, blot and count pellets

Table 3.14 Preparation and Component Dilutions for the Pre-precipitated Anti-T_3 Reagent

Reagent Component	Volume (ml)	Dilution		
		Stock Reagent	Working Reagent	RIA Incubate
DAS	11.56	1:1.73	1:5.2	1:80
NSS	0.28	1:71	1:213	1:3,300
Anti-T_3*	0.04	1:50,000	1:150,000	1:2,300,000
Diluent	8.12			
Total	20.00			

* x 100 dilution of neat antiserum.

Precision profiles for a series of standard curves using different dilutions of primary antibody were examined to optimise the anti-T_3 dilution (Figure 3.19).

In the pre-precipitated form, the affinity constant (K_a) of the primary antiserum was calculated by Scatchard analysis as 8 $x10^{11}$ l/mol.

Standards were prepared from a 1 mmol/l stock T_3 solution (Section 2.2.1) by dilution in HEPES buffer with 5% gelatine and stored at -20°C (400, 200, 100, 50, 25 pmol/l). These were diluted 12.5-fold in the RIA protocol. As an additional check on the accuracy of the dilutions, a suitable intermediate dilution (approx. 2 nmol/l) in charcoal-stripped serum was measured for T_3 in the serum total T_3 assay (Section 2.2.2).

3.6.2 Precision and Sensitivity of the T_3 RIA

The mean within-assay precision profile calculated from duplicates of standards and dialysates for 12 assays is shown in Figure 3.20. The working range of the RIA was 2 - 32 pmol/l. The sensitivity of the assay, defined as 2.5 SD above zero from 40 replicates of the zero standard was 0.5 pmol/l. The between-assay reproducibility of the RIA was assessed using two buffer pools stored with 5% gelatine. In 25 assays, the CVs at 4.3 and 9.0 pmol/l were 11.4% and 7.0% respectively.

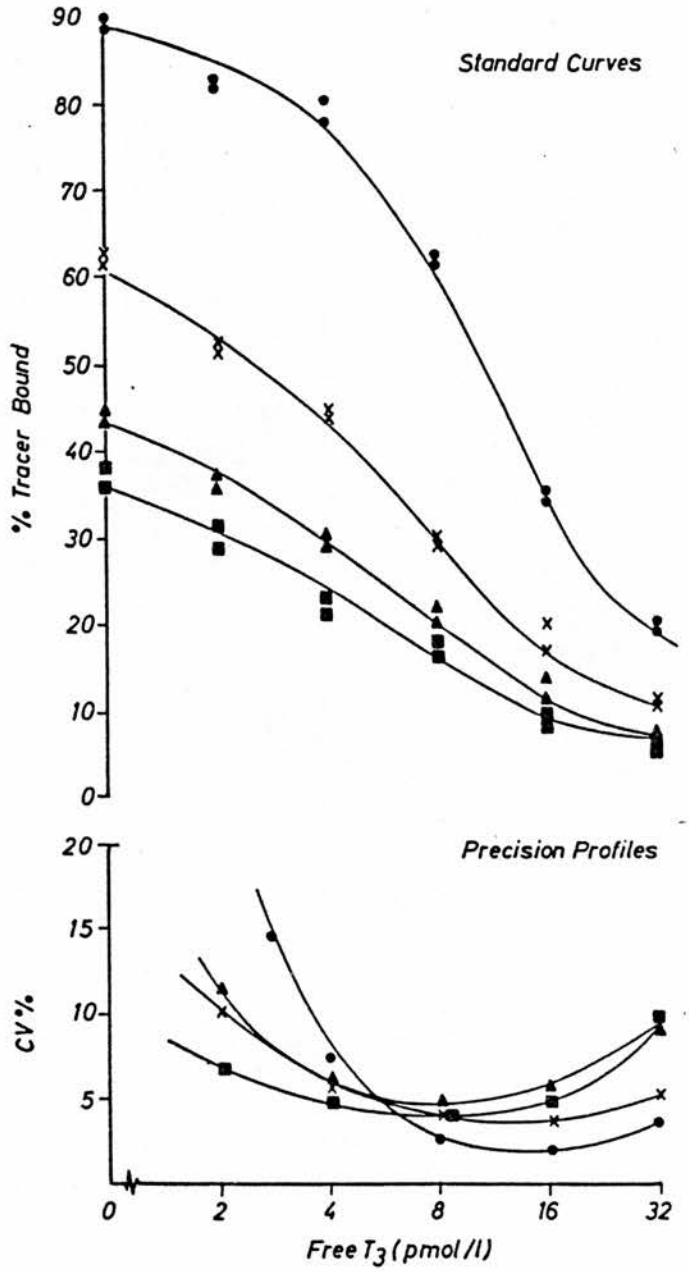


Figure 3.19 Standard Curves and Precision Profiles at Different Final Dilutions of Anti-T_3 Antiserum.

Final dilutions were: ●—● 1:1x10^6; x—x 1:2x10^6; ▲—▲ 1:3x10^6; ■—■ 1:4 x 10^6.

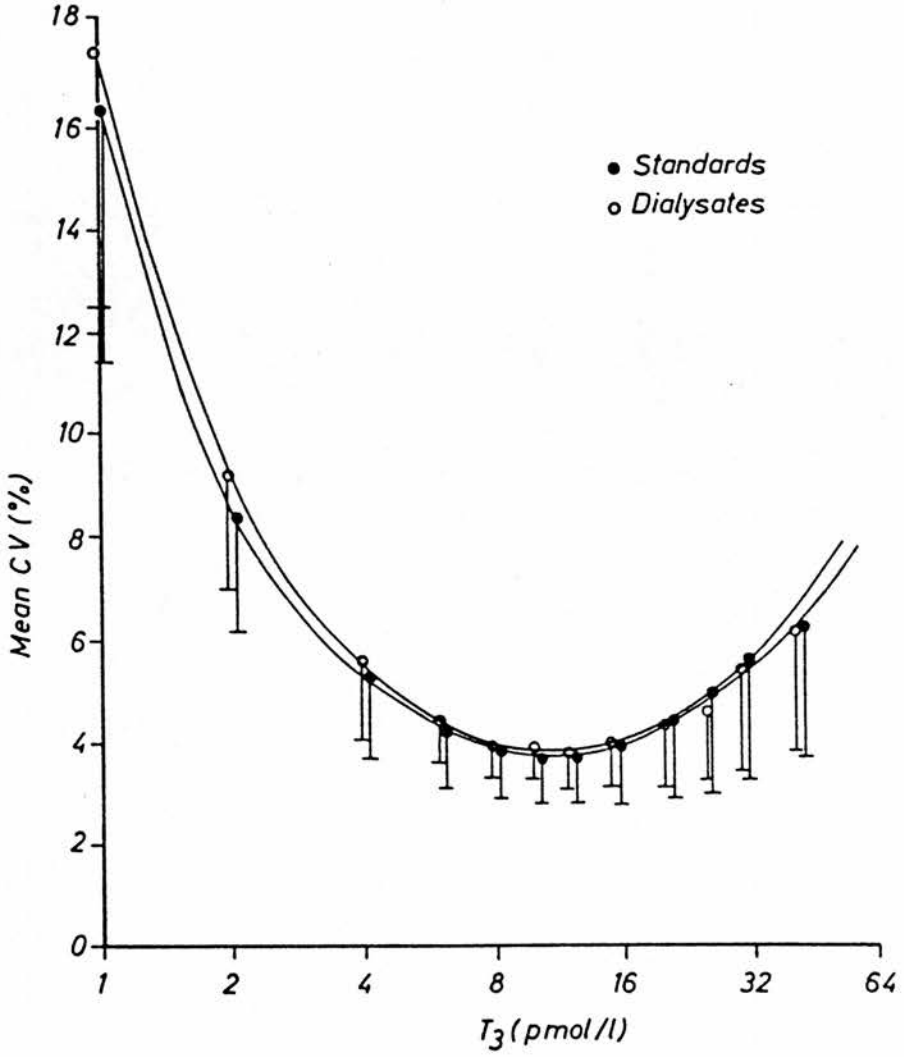


Figure 3.20 Within-assay Precision Profiles (Mean±SD, n = 12) for the RIA of T_3 in Standards and Dialysates.

3.6.3 Optimisation of the Equilibrium Dialysis Procedure for Free T_3 Measurement

There was little difference in the fT_3 values of serum and the time to reach equilibrium in the dialysis step using either Visking or Spectrapor dialysis membranes, Figure 3.21. Plateau values for fT_3 were reached after 16-18 h dialysis at 37°C. An overnight dialysis with Visking membranes for 18-24 h at 37°C was used in subsequent assays.

A greater effect on fT_3 compared to fT_4 values was observed when serum was pre-diluted with HEPES buffer prior to dialysis (Figure 3.22). This is in agreement with the theoretical study of Kamikubo et al., 1984. On average, dialysis using a 1:10 dilution of serum versus 1:20 produced 15% higher fT_3 values. Slightly higher values were produced using less dilute serum. Further dialysis runs were performed using a 1:10 dilution of serum i.e. 100 µl of serum per dialysis cell.

3.6.4 Precision of the Free T_3 Dialysis-RIA

The mean precision profile for the T_3 RIA using serum dialysates was very similar to that for standards (Figure 3.20). The within-run precision for dialysis cells assessed by dialysing a euthyroid and a hyperthyroid sample in 10 cells each was 6.7% and 7.6% at fT_3 values of 3.3 and 25.7 pmol/l, respectively. The variation in values using two dialysis cells per patient sample was

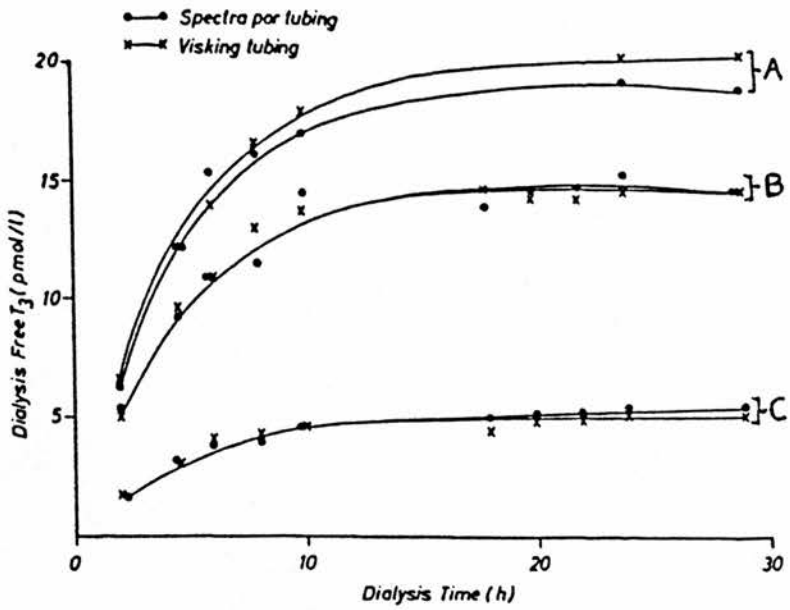


Figure 3.21 Free T_3 Equilibrium Dialysis Time-course for Three Serum Samples (A,B,C).

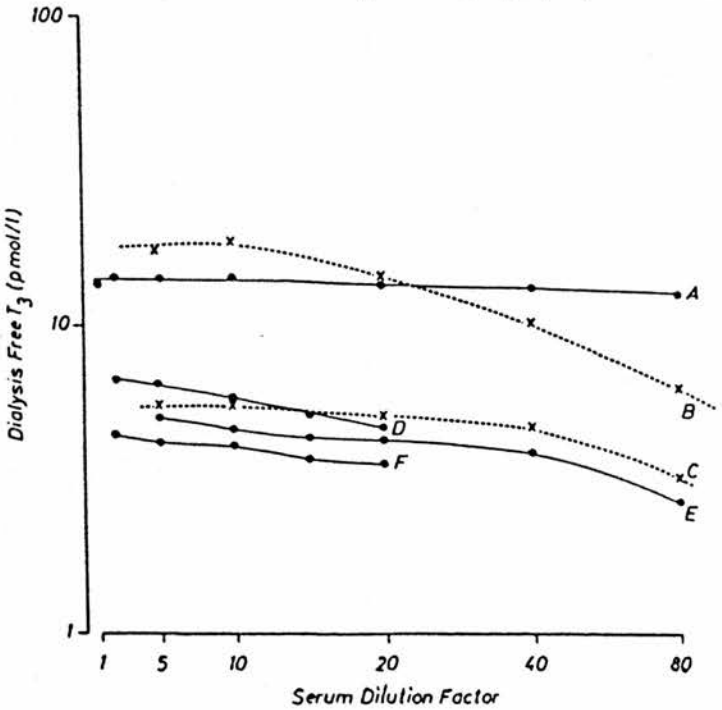


Figure 3.22 The Effect of Sample Pre-dilution Prior to Dialysis-RIA of Free T_3.

Sera from a thyrotoxic patient (A), euthyroid patients (D,E,F), pooled normal serum (C) and a serum pool with added T_3 (B) were analysed.

7.2% (range 1.3-6.0 pmol/l) and 8.3% (range 6.0 - 36.0 pmol/l). Because of this satisfactory within-assay precision, patients' samples used in the method validation were dialysed in singleton.

The overall between-assay precision was calculated in 12 dialysis runs from the analysis of pooled normal serum and this pool with added T_3. These pools were stored at -20°C. The CVs were 7.9% at 6.7 pmol/l and 10.3% at 12.2 pmol/l. The variation in fT_3 values for RAC sera from patients over 10 assays is summarised in Table 3.15.

Table 3.15 Dialysis-RIA Free T_3 Between-assay Precision from RAC Sera

n	Range (pmol/l	Mean (pmol/l) 1	2	SD (pmol/l)	t	CV(%)
27	2.3- 7.7	4.58	4.54	0.55	0.25	12.0
46	6.0-39.0	15.62	16.46	1.68	-2.4	10.5

3.6.5 Validation of the Reference Free T_3 Method

Sera collected from 162 patients attending the thyroid clinic (Section 4.1) were analysed for fT_3 by dialysis-RIA. Samples from patients in different diagnostic categories were dispersed in separate dialysis runs and their fT_3 results are shown in Figure 3.23.

All patients with overt hyperthyroidism and 3 of the 4 with subclinical hyperthyroidism had raised

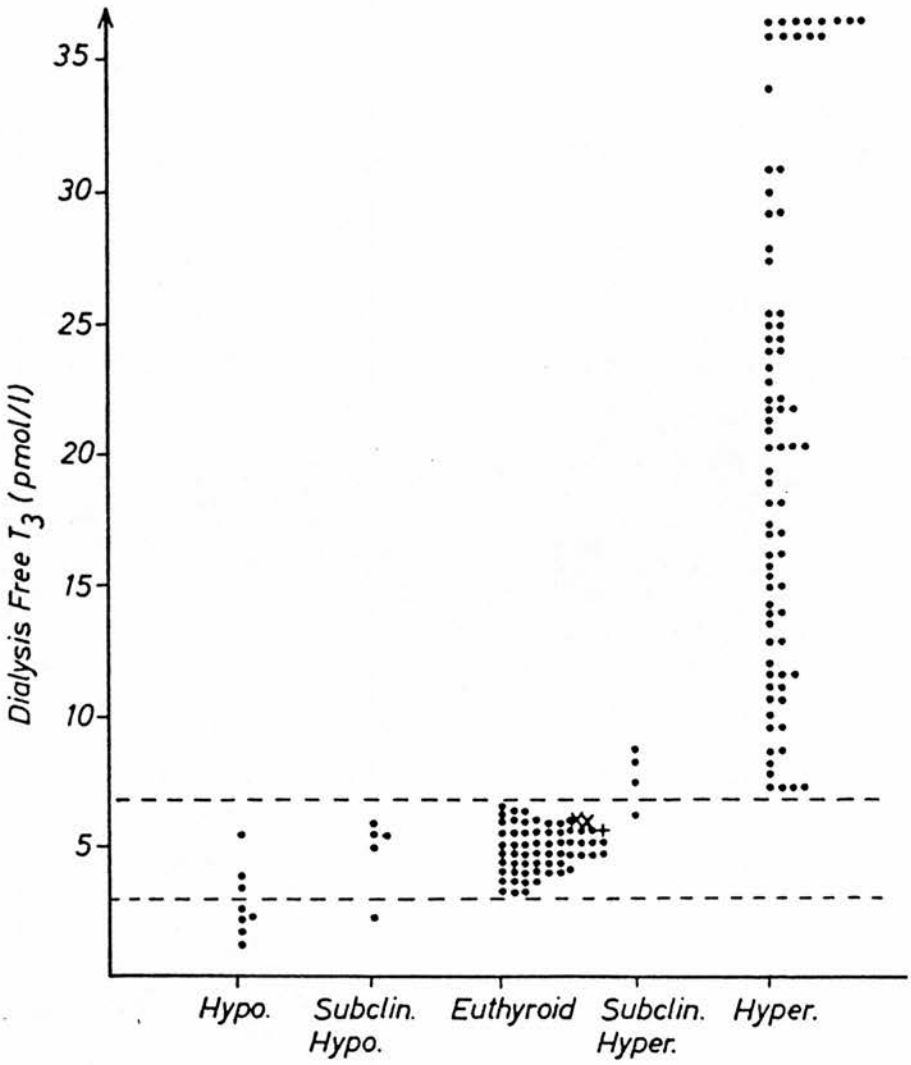


Figure 3.23 Free T_3 Concentrations by Dialysis-RIA in 162 Patients from a Thyroid Clinic.

Two euthyroid patients were taking an OCP (x) and one had high affinity binding of T_4 to albumin (+).

fT_3 concentrations. Euthyroid patients taking the OCP and one patient with abnormal T_4 binding to albumin had normal fT_3 concentrations. The 95% reference range derived from the 63 euthyroid patients was 3.2-6.7 pmol/l. Normal fT_3 results were found in a number of hypothyroid patients.

The reference values for this method agreed well with those reported for several indirect fT_3 assays (Table 1.3) but were generally lower than those quoted for other direct methods (Table 1.4 and 3.16).

Table 3.16 The Performance and Reference Values of Recently Described Direct Free T_3 Reference Methods

Method	This Study	Liewendahl et al., 1984	Weeke et al., 1986
	Equilibrium dialysis + RIA	Equilibrium dialysis + RIA	Ultra-filtration + RIA
Overall serum dilution	1:22	1:54	1:1
RIA separation system	Pre-precipitated double-antibody	Dextran-coated Charcoal	PEG precipit-ation
Intra-assay CV	7%	5%	9%
Inter-assay CV	10%	-	10%
Sensitivity (pmol/l)	0.5	-	-
Reference Range (pmol/l)	3.2-6.7	4.3-10.3	5.4-12.1

Chapter 4

A LABORATORY AND CLINICAL EVALUATION OF FREE THYROID HORMONE AND SENSITIVE TSH MEASUREMENTS IN PATIENTS WITH SUSPECTED THYROID DISEASE

4.1. SAMPLES USED IN THE STUDY

Serum samples were collected from 285 consecutive patients referred to a thyroid clinic either for further investigation of suspected thyroid disease or for ^{131}I-treatment of hyperthyroidism. There were 247 females and 38 males (age range 13-80 years, mean 47.6). The diagnosis in each case was made on clinical grounds and using results for total T_4, T_3, basal TSH (in-house RIA) and the TSH response 20 min after the intravenous administration of 200 µg TRH. The iodine-131 uptake test, thyroid scans and tests for thyroid autoantibodies were also used to assist in the diagnosis. The distribution of thyroid pathology in the group is shown in Table 4.1.

Table 4.1. Distribution of Thyroid Pathology in 285 Thyroid Clinic Patients

Thyroid Pathology	n
Graves' disease	123
Toxic multinodular goitre	16
Toxic solitary nodule	8
Multinodular goitre	27
Subclinical Graves' disease	9
Non-toxic solitary nodule	14
Simple goitre	6
Cold nodule or cyst	6
Ophthalmic Graves' disease	2
Hashimoto's thyroiditis	20
Primary atrophic hypothyroidism	5
No thyroid pathology	49

Eight of these patients were known to be taking an oestrogen containing oral contraceptive (OCP) and one was pregnant.

The biochemical tests were used to assign patients to diagnostic categories as shown in Table 4.2.

Table 4.2. The Distribution of Patients in Five Diagnostic Categories

Category	Test Results	n
Hyperthyroid	Absent response to TRH High total T_4 and/or T_3	147
Subclinical Hyperthyroid	Absent response to TRH Normal total T_4 and T_3	16
Euthyroid	Normal response to TRH Basal TSH not raised Normal total T_4 and T_3	97
Subclinical Hypothyroid	Increased basal TSH and exaggerated response to TRH. Normal total T_4	16
Hypothyroid	Increased basal TSH and exaggerated response to TRH. Low total T_4	9

The reference ranges used for TSH (RIA) pre- and post-TRH, total T_4 and T_3 were those described in Chapter 2. A TSH increment at 20 min after TRH of less than 1.0 mU/L above the basal value was considered an absent response. Patients with normal total thyroid hormone concentrations but either an absent TSH response to TRH or a raised basal TSH concentration were described as having subclinical hyperthyroidism or subclinical hypothyroidism, respectively. Hyperthyroid patients with normal total T_4 but

raised total T_3 concentrations were described as T_3-thyrotoxic. There were 11 such patients in this series. Three patients with normal TRH tests who were categorised as euthyroid had marginally increased total T_4 (no thyroid pathology) or T_3 levels (one with a multinodular goitre and one with ophthalmic Graves' disease).

The first 200 samples collected in this series were used in the evaluation of the free thyroid hormone kits (Section 4.2 and 4.3) and both TSH IRMA methods. In the evaluation of TSH IRMA methods (Section 4.5), the subclinical disease categories were supplemented with additional samples. All of the 285 samples were used to evaluate a new thyroid function test strategy (Section 4.6). Sera from a separate series of 116 consecutive patients were used in the evaluation of the SimulTRAC dual-analyte assay (Section 4.4). All sera were split into three portions and stored at -20°C until assay.

4.2 FREE T_4 MEASUREMENT BY ANALOGUE RIA

This section deals with the evaluation of various analogue RIA kits in thyroid clinic patients. Data from other groups of patients are described in subsequent chapters.

4.2.1 Comparison of Free T_4 RIA Procedures

The manufacturers' protocols are summarised in Table 4.3. All assays were performed using an automatic

Table 4.3. Protocols for Free T_4 and Free T_3 by Analogue RIA

	Amerlex		Amerlex Magnetic		Becton Dickinson		Coat A Count		Corning Magic
	fT_4	fT_3	fT_4	fT_3	fT_4	fT_3	fT_4	fT_3	fT_4
Sample vol, µl	100	100	100	100	50	100	50	100	50
Pipetting steps	2	2	2	2	1	1	1	1	2
Incubation at 37°C, h	1	2	1	2	1.5	2.5	1	3	1
Wash step	No		No		Yes		No		No
Separation method	Centrifugation of Amerlex particles		Magnetic 1st antibody		Coated tube		Coated tube		Magnetic 1st antibody

diluter dispensing sample with tracer in one step within 10 min to minimise effects of drift, particularly in the coated-tube assays. All tests were performed in duplicate, with a maximum of 50 duplicates per assay. The Amerlex Magnetic (Amerlex-M) kit was introduced after the original Amerlex kit. Both kits contain identical antibody, analogue and standards but the Amerlex-M kit also contains an "albumin blocker" which is claimed to remove interference in the fT_4 assay due to changes in albumin concentration.

4.2.2 Within-assay and Between-assay Precision

The mean within-assay precision profiles (from duplicates of samples) for each kit and the reference dialysis method from 15 assays are shown in Figure 4.1. The relatively poor precision of the reference method represents the combination of imprecision from the dialysis and RIA steps. The Amerlex and Amerlex-M assays showed the best profiles whilst the Corning Magic kit showed poor precision at low values. For practical purposes all of the kits had adequate precision between 3 and 100 pmol/l.

The average number of tests per run where there was less good agreement between duplicate tubes ("duplicate error") was compared. This provided an important indicator of within-assay precision, since "duplicate errors" are not included in the derivation of

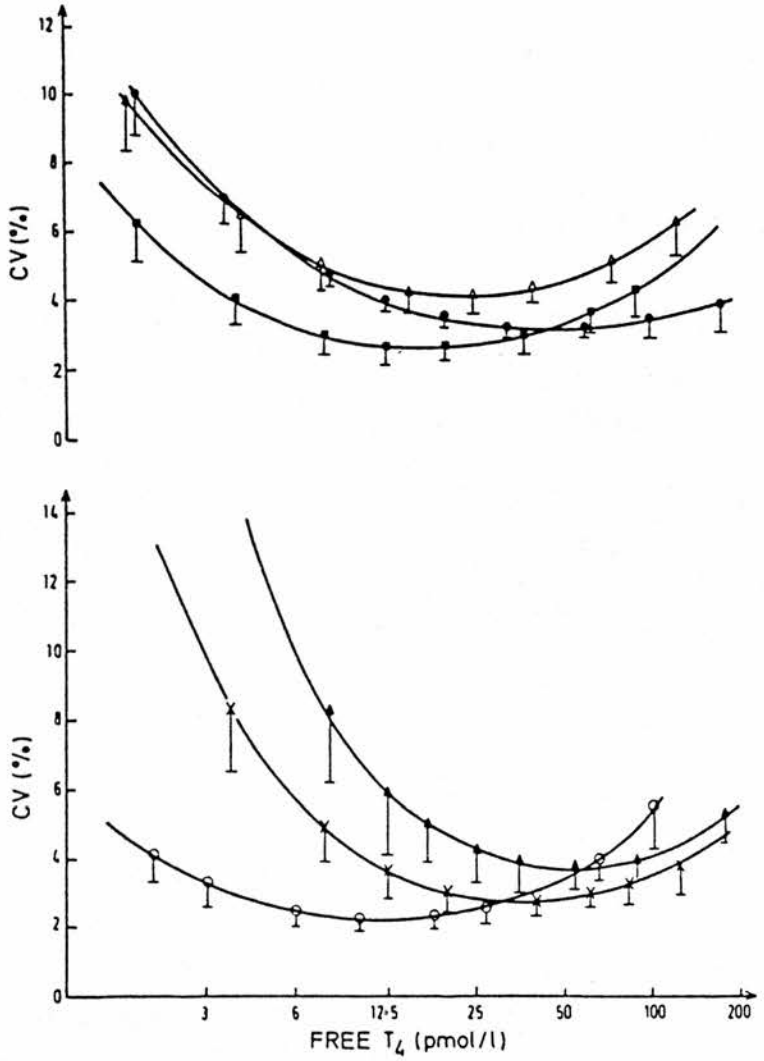


Figure 4.1 Mean Within-assay Precision Profiles for 15 Assays of Free T4 by Each Method.

Reference intervals (pmol/l) were:
11-23 Coat A Count (Δ),
8-17 Becton Dickinson (●),
8-17 Equilibrium dialysis (▲),
10-22 Amerlex-M (■),
10-21 Amerlex (o),
17-30 Corning (x).

Bars indicate 1 SD.

the within-assay precision profile. A duplicate error was indicated if the variance ratio of the replicate counts to the mean counts exceeded the arbitrary value of 20 (Baxter, 1982). For each kit the mean percentage of duplicate errors per run was: Amerlex 27%, Amerlex-M 14%, Becton Dickinson 18%, Coat A Count 24%, Corning Magic 13%. This represents the number of tests where the replicate values required further scrutiny to determine if re-analysis was necessary. The kits using magnetic separation had fewer duplicate errors than the other separation systems. The beads used in the Amerlex separation step did not form a robust compact pellet after centrifugation leading to a loss of antibody at the decanting stage and thus to imprecision and an unacceptably high number of samples requiring re-analysis. The wash step employed in the Becton Dickinson assay may account for the lower number of duplicate errors compared to the Coat A Count kit.

The between-assay precision was assessed using two human Commercial control sera (RIATRAC II and III, Becton Dickinson Lot No. HL4000) and a low serum pool (Table 4.4). In addition, a patient's sample from the previous run was re-analysed in the next assay (repeat analysis control, RAC) and, for values within the range 5-50 pmol/l, the precision (CV) calculated from the two results on each RAC from 10 assays was: Amerlex 9%,

Table 4.4. Between-assay Precision of the Free T_4 Kits

FT_4	Amerlex	Amerlex Magnetic	Becton Dickinson	Coat A Count	Corning Magic
Low Pool					
n	13	16	15	10	19
Mean, pmol/l	7	7	7	8	13
SD, pmol/l	0.3	0.3	0.6	0.6	0.8
CV, %	5	5	9	7	6
RIATRAC II					
n	17	19	15	10	24
Mean, pmol/l	12	13	9	11	19
SD, pmol/l	0.6	0.6	0.8	0.8	0.9
CV, %	5	5	9	7	5
RIATRAC III					
n	15	16	16	10	24
Mean, pmol/l	35	31	21	28	38
SD, pmol/l	1.4	2.2	2.1	2.0	1.9
CV, %	4	7	10	7	5

Amerlex-M 5%, Becton Dickinson 9%, Coat A Count 7%, Corning Magic 6%. The magnetic separation (Amerlex-M, Corning Magic) and Amerlex fT4 kits had better between-assay precision than the coated-tube assays and this has been confirmed in other studies (Wilke, 1982; Witherspoon et al., 1985; Jackson & Ekins, 1986).

4.2.3 Investigation of Assay Drift

One sample in each of 10 assays was analysed at the beginning and the end of batches containing 50 duplicate tubes. There was no significant drift in the Amerlex, Amerlex-M and Corning Magic assays but both coated-tube assays showed a positive drift of 7 - 8% ($p<0.05$) contributing to their poorer precision. Wilke (1982) also detected drift using the Coat A Count kit and noted that this assay had not reached equilibrium in the recommended incubation time.

4.2.4 Standardisation Compared to the Reference Method

The standards provided with each kit were analysed in the same assay by the reference fT4 dialysis method. The mean dialysis values from 5 runs were compared with the quoted values (Figure 4.2). Regression analysis showed that the Amerlex (or Amerlex-M) and Becton Dickinson standards were targeted in agreement with the reference method whereas Coat A Count and Corning Magic standards were assigned values significantly higher ($p<0.001$) than those measured by dialysis. This is in

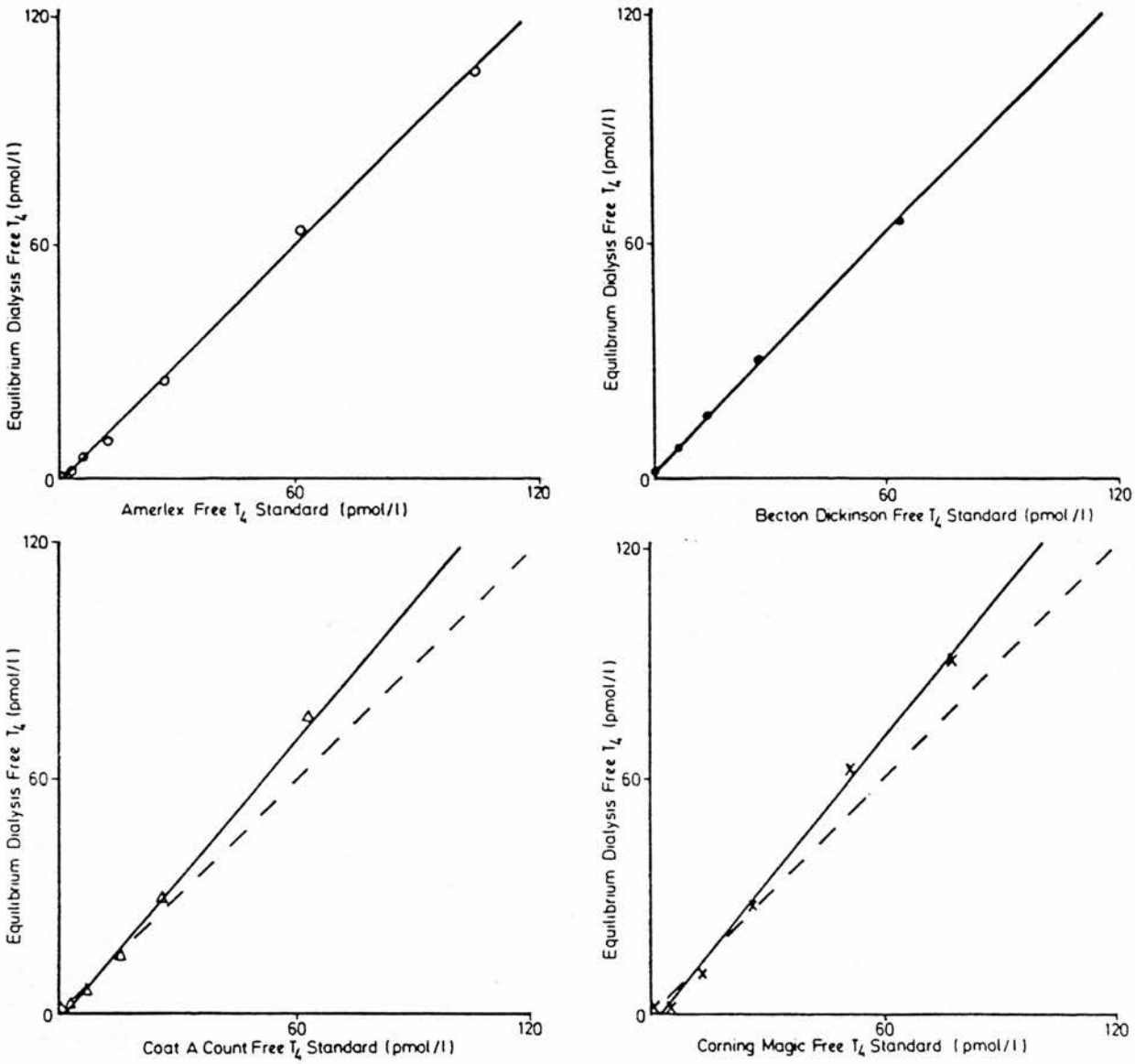


Figure 4.2 Free T_4 Kit Standards Measured by Equilibrium Dialysis.

Regression equations for fT_4 values were:
Dialysis = 1.0 Amerlex -0.5,
Dialysis = 1.0 Becton Dickinson +1.0,
Dialysis = 1.2* Coat A Count -2.0,
Dialysis = 1.2* Corning Magic -2.6.

*Significant differences from y = x (---).

broad agreement with the findings of the recent DHSS survey of fT_4 methods with the exception of the Becton Dickinson standards which were found to be targetted too high in that study (Jackson & Ekins, 1986).

4.2.5 Comparison of Free T_4 Results with the Reference Method

For the first 162 of the 200 study patients, fT_4 values by analogue RIA were compared with those by dialysis (Section 3.5.9). Free T_4 values <100 pmol/l were used in the comparison and results for four of the kits are shown in Figure 4.3 (values by Amerlex-M did not differ significantly from Amerlex fT_4 values). Correlation coefficients of $r>0.94$ were obtained for all the analogue methods.

Free T_4 values by Amerlex, Amerlex-M and Becton Dickinson kits, did not differ significantly from the reference method but Coat A Count and Corning Magic values at low concentrations fell below the regression line drawn, indicating that this relationship was not rectilinear. In general, the Coat A Count kit gave lower values than those by dialysis at high fT_4 concentrations consistent with the standardisation difference demonstrated. Corning Magic however, gave higher values throughout than dialysis, resulting in the higher reference range for this kit. (Several values which were greater than the top standard (80 pmol/l) provided with the Corning Magic kit were not included in Figure 4.3).

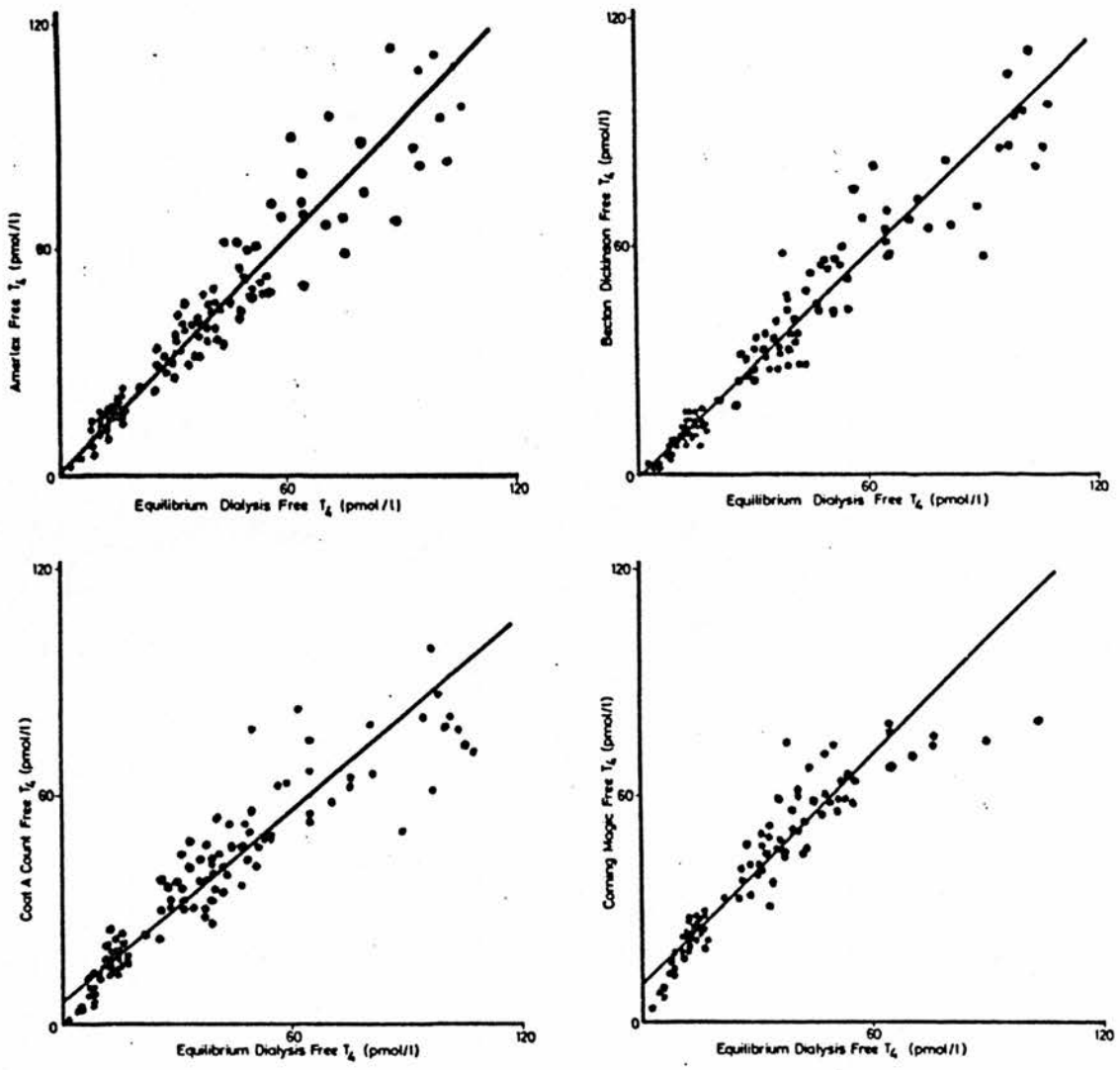


Figure 4.3 Comparison of Free T4 Values by Analogue RIA and Equilibrium Dialysis in Patients' Samples.

Regression equations (y = mx + c) were:

Amerlex = 1.04 dialysis +1.3,
Becton Dickinson = 0.97 dialysis -0.5,
Coat A Count = 0.84* dialysis +5.8*,
Corning Magic = 1.00 dialysis +10.0*.

(*Significant differences, $p<0.001$, from y=x)
Amerlex-M = 0.97 dialysis +2.3 (data not shown).

This is not consistent with the negative bias of the kit standard values compared with those measured by dialysis and suggests that some other feature of this kit e.g. the analogue or the matrix of the standards is responsible for the difference in accuracy using patient samples.

Similar relationships have been reported for the Coat A Count versus the Amerlex kit (Kubasik et al., 1983) and, more recently, for the Amerlex, Becton Dickinson and Coat A Count kits versus direct dialysis (Jackson & Ekins, 1986). Discrepant results for standards and samples with respect to dialysis were also found for the non-magnetic Corning analogue RIA in the latter report.

4.2.6 Diagnostic Accuracy in Patient Samples

Despite the differences in accuracy, all of the fT4 kits performed equally well in categorising patients. Results by four of the kits used for 200 sera from the thyroid clinic series are shown in Figure 4.4. The Amerlex-M kit categorised the patients in an identical manner to the Amerlex kit and therefore this data is not shown.

The reference intervals (mean±2SD) derived from 62 euthyroid patients were: Amerlex 10-21; Amerlex-M 10-22; Becton Dickinson 8-17; Coat A Count 11-23; Corning Magic 17-30, pmol/l. All but one kit correctly classified the two euthyroid women with raised total T4 due to the OCP. However, since only two such patients

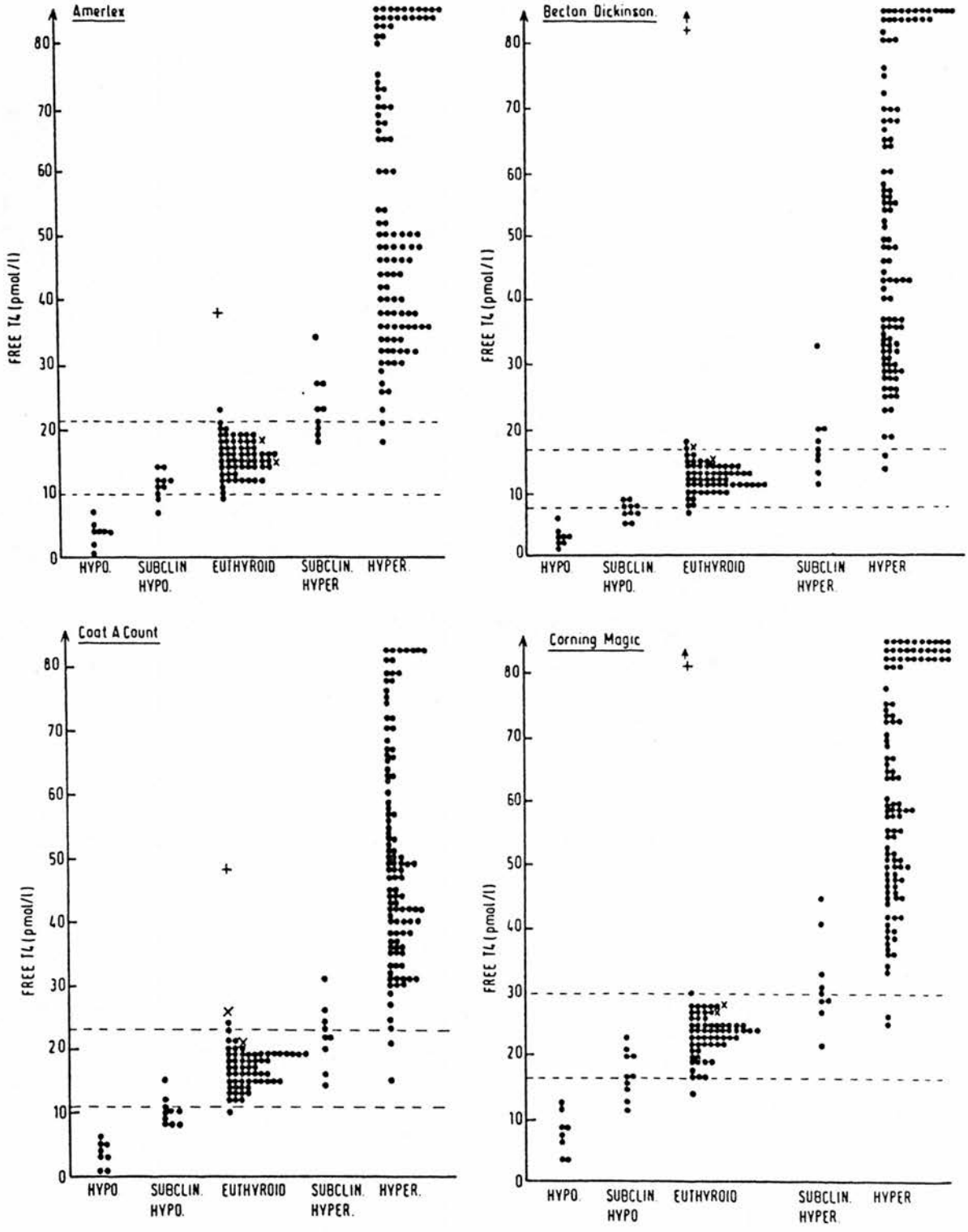


Figure 4.4 Free T_4 Values by Analogue RIA Kits in 200 Patients from a Thyroid Clinic.

Two patients were taking oral contraceptives (x); one patient with high fT_4 had an abnormal high affinity albumin (+).

were studied, no conclusions could be drawn on the kits' performance in this situation. One euthyroid man had an abnormal high affinity albumin present in his serum (Section 5.2.2); this gave raised values with all the fT_4 kits, although fT_4 was normal by equilibrium dialysis. His results were excluded when calculating the reference intervals above.

Two patients with overt hyperthyroidism were misclassified by all the kits: one had T_3-thyrotoxicosis, the other had marginal elevations in total T_4 and T_3 with a normal fT_4 by the kits and by dialysis.

In the subclinical groups, a proportion of fT_4 results were outside the reference intervals. Out of the 19 patients in these groups (Figure 4.4), abnormal fT_4 results were found in a maximum of 7 to 10 patients, depending on the kit used. Many of these patients, therefore, had fT_4 levels more consistent with overt thyroid disease. This improved resolution of borderline cases contrasts with the findings of Braun *et al.*, 1983.

In conclusion, although the diagnostic accuracy was comparable for all the analogue kits assessed as found by other workers (Wilke 1982; Nasralla *et al.*, 1983; Witherspoon *et al.*, 1984; Jackson & Ekins, 1986), better precision and analytical accuracy were obtained with the Amerlex-M fT_4 kit. Since this kit only became available

in 1984 some of the earlier clinical studies in this thesis were carried out using Amerlex fT_4 kits. It should also be noted that since 1985 the Coat A Count kit has been modified further to negate any albumin effects.

4.3 FREE T_3 MEASUREMENT BY ANALOGUE RIA

4.3.1 Comparison of Free T_3 RIA Procedures

The manufacturers' protocols for the fT_3 assays evaluated are outlined in Table 4.3. All assays were limited to 50 duplicate tubes. Unlike fT_4, no drift was found for any of the fT_3 assays. This may be due to the greater incubation times employed for the latter (2-3 h).

Amersham International replaced their Amerlex fT_3 kit with the magnetic version late in 1984. An "albumin-blocker" was not incorporated but the method of standardisation was changed. A computer model is used by the manufacturers to predict fT_3 concentrations in the standards from the measured total T_3 concentrations. An improved model was used in the Amerlex-M kit.

4.3.2 Within-assay and Between-assay Precision

The mean within-assay precision profiles for the fT_3 kits and the reference dialysis method for 12 assays are shown in Figure 4.5. The profile for the reference method compared favourably with the kit methods despite being more technically demanding. The Amerlex method with centrifugal separation had the lowest profile but all methods had a working range (<10% CV) of 2-40 pmol/l.

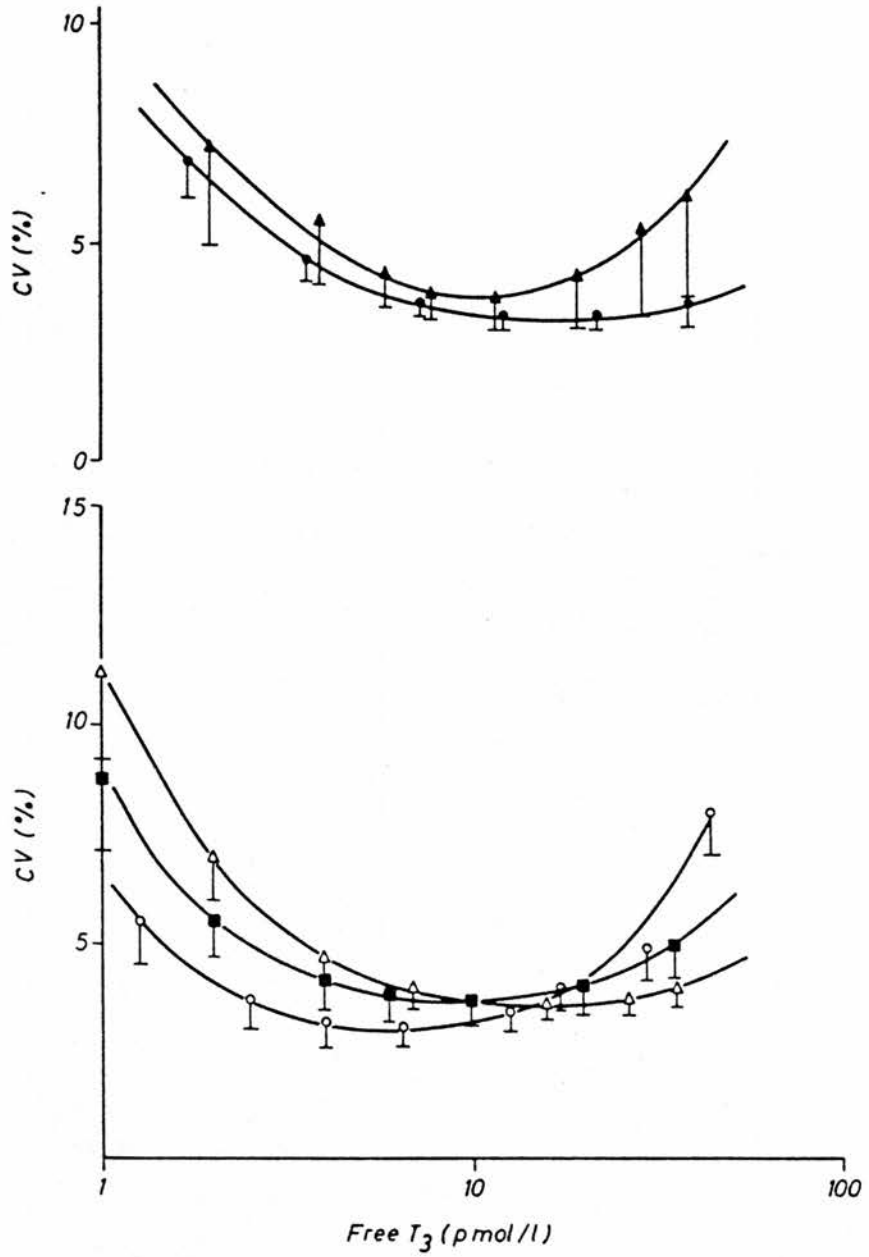


Figure 4.5 Mean Within-assay Precision Profiles for 12 Assays of Free T_3 by Each Method.

Reference intervals (pmol/l) were:
3.2-6.7 equilibrium dialysis (▲),
5.3-10.0 Becton Dickinson (●),
3.0-6.9 Coat A Count (Δ),
4.2-7.8 Amerlex-M (■),
4.0-7.8 Amerlex (o).

Bars indicate 1 SD.

The mean percentages of duplicate errors per assay were: Amerlex 11%, Amerlex-M 6%, Becton Dickinson 4%, Coat A Count 12%. Again the use of the magnetic separation improved the performance of the Amersham kit in this regard. There were fewer duplicate errors with all the fT_3 kits compared to their fT_4 counterparts (Section 4.2.2). The larger sample volumes used in the coated-tube fT_3 kits and the longer incubation times may account for this.

The between-assay precision of the kits was assessed using either human commercial control sera (RIATRAC I, II, III Lot No. HL4000, from Becton Dickinson) or serum pools used in the quality control of the total T_3 assay (Table 4.5). The precision (CV) calculated from RAC results in the range 3-25 pmol/l from 10 assays was: Amerlex 6.9%, Amerlex-M 6.2%, Becton Dickinson 7.0% and Coat A Count 8.7%. The Amerlex-M fT_3 kit had the best between-assay precision of the kits.

4.3.3 Standardisation Compared to the Reference Method

To determine the accuracy of the standardisation of the analogue kits, kit standards were analysed by fT_3 dialysis-RIA. The mean dialysis values from 3 runs were compared with the values quoted by the manufacturers (Figure 4.6). The assigned fT_3 values over-estimated the fT_3 values measured by dialysis-RIA in all kits.

Table 4.5 Between-assay Precision of the Free T_3 Kits

FT_3	Amerlex	Amerlex Magnetic	Becton Dickinson	Coat A Count
Control	Low Pool	Low Pool	RIATRAC I	RIATRAC I
n	15	18	19	10
Mean, pmol/l	2.8	3.0	4.4	2.1
SD, pmol/l	0.19	0.13	0.30	0.18
CV, %	6.7	4.2	6.9	8.8
Control	Medium Pool	Medium Pool	RIATRAC II	RIATRAC II
n	15	18	18	10
Mean, pmol/l	7.7	8.2	8.5	4.5
SD, pmol/l	0.58	0.50	0.64	0.42
CV, %	7.5	6.1	7.5	9.3
Control	High Pool	High Pool	RIATRAC III	RIATRAC III
n	15	17	20	10
Mean, pmol/l	18.4	17.4	13.4	10.3
SD, pmol/l	0.86	0.66	0.88	0.85
CV, %	4.7	3.8	6.6	8.3

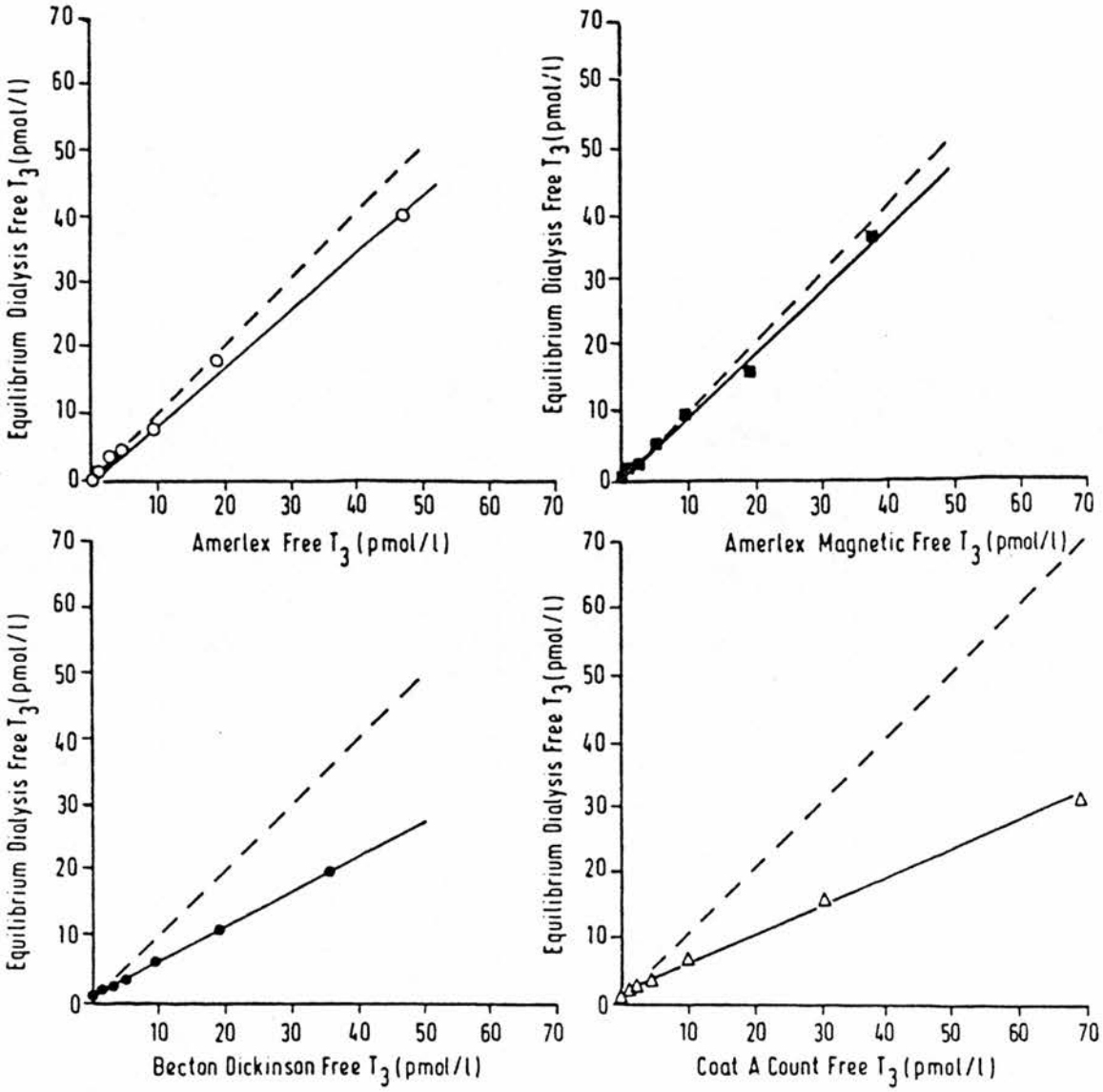


Figure 4.6 Free T_3 Kit Standards Measured by Equilibrium Dialysis

Regression equations for fT_3 values were:

Dialysis = 0.84* Amerlex +0.7,
Dialysis = 0.92* Amerlex-M +0.4,
Dialysis = 0.54* Becton Dickinson +0.6,
Dialysis = 0.45* Coat A Count +1.2.

*Significant differences from y=x (---).

However, the discrepancy was most marked with the coated-tube methods. At high concentrations, values assigned to the Amerlex standards were higher compared to dialysis-RIA than those assigned to Amerlex-M standards. This is consistent with the known re-targetting of the Amerlex-M standards by the manufacturer.

4.3.4 Comparison of Free T_3 Results with the Reference Method

Free T_3 values measured by dialysis-RIA (Section 3.6.5) and the analogue kits were compared for the first 162 patients in the study. Regression analysis was performed for pairs of fT_3 values less than 32 pmol/l (Figure 4.7). Correlation coefficients of $r>0.92$ were obtained for all of the analogue methods. A lack of linearity at low concentrations was evident for three of the kits which had a significant y-intercept. Although fT_3 values by Amerlex, Amerlex-M and Becton Dickinson differed slightly, Coat A Count values were much lower than dialysis values particularly in hyperthyroid patients. There was much better agreement between Coat A Count and dialysis values in sera from euthyroid patients (Figure 4.7) and Wang *et al*. (1985) have confirmed this using an indirect ultrafiltration method.

The regression analysis for the two Amerlex fT_3 kits is shown in Figure 4.8. The relationship was not rectilinear with the magnetic version giving lower

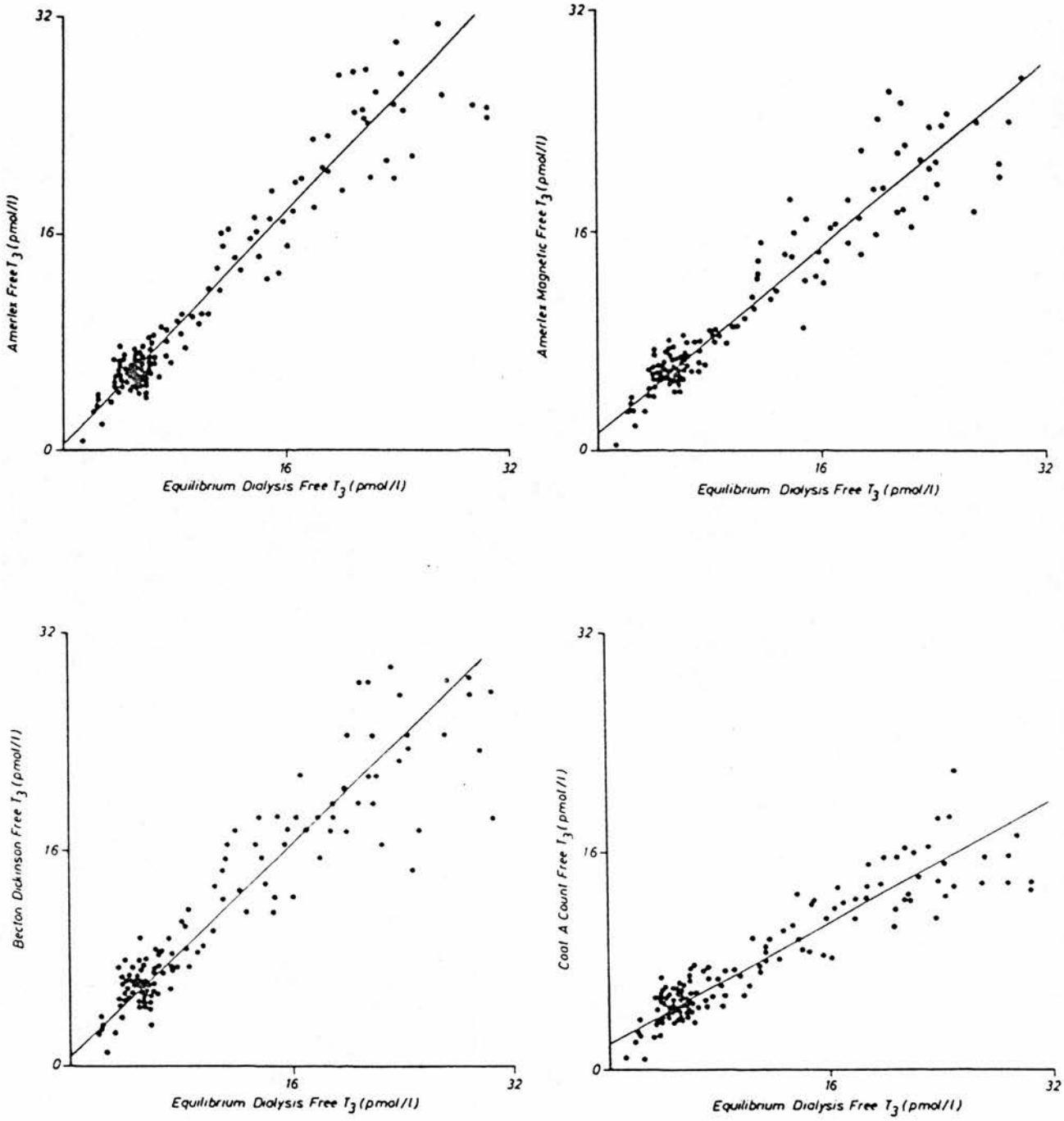


Figure 4.7 Comparison of Free T_3 Values by Analogue RIA and Equilibrium Dialysis in Patients' Samples.

Regression equations (y=mx+c) were:

Amerlex	= 1.06* Dialysis +0.5,
Amerlex-M	= 0.85* Dialysis +1.4*,
Becton Dickinson	= 0.98* Dialysis +2.4*,
Coat A Count	= 0.56* Dialysis +1.8*.

(*Significant differences, $p<0.001$, from y=x).

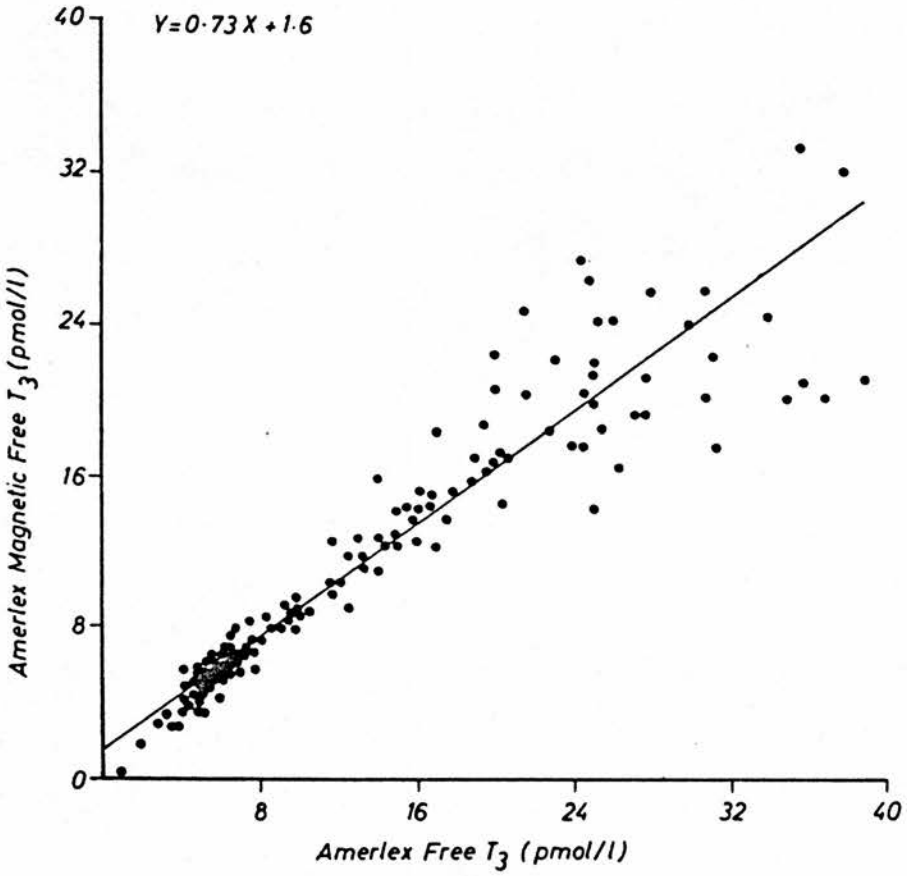


Figure 4.8 Regression Analysis of Free T_3 Values by Amerlex and Amerlex Magnetic Analogue RIA.

fT_3 values at high concentrations in keeping with the change in standardisation by the manufacturer.

The coated-tube kits produced the greatest discrepancy in relation to dialysis values comparing patient samples and standards. The assigned standard values were approximately two times higher than those measured by dialysis for both coated-tube kits. However, in patient samples, the Becton Dickinson kit produced similar values to dialysis whereas Coat A Count values were generally lower than dialysis values. This suggests that standards and patient samples behave differently in these methods. (A similar discrepancy was observed for the Corning Magic fT_4 kit). This effect may be due to the different analogues and standard matrices used by different manufacturers.

4.3.5 Diagnostic Accuracy in Patient Samples

The fT_3 kits produced a greater disparity in the categorisation of the 200 patients than the fT_4 kits. Results in the five patient categories for each kit are shown in Figure 4.9.

The reference intervals (mean±SD) derived from 62 euthyroid patients were: Amerlex 4.0-7.8; Amerlex-M 4.2-7.8; Becton Dickinson 5.3-10.0; Coat A Count 3.0-6.9, pmol/l. In the two euthyroid patients taking the OCP, fT_3 values were normal by all except the Coat A Count kit; fT_4 (Coat A Count) was also raised in this patient.

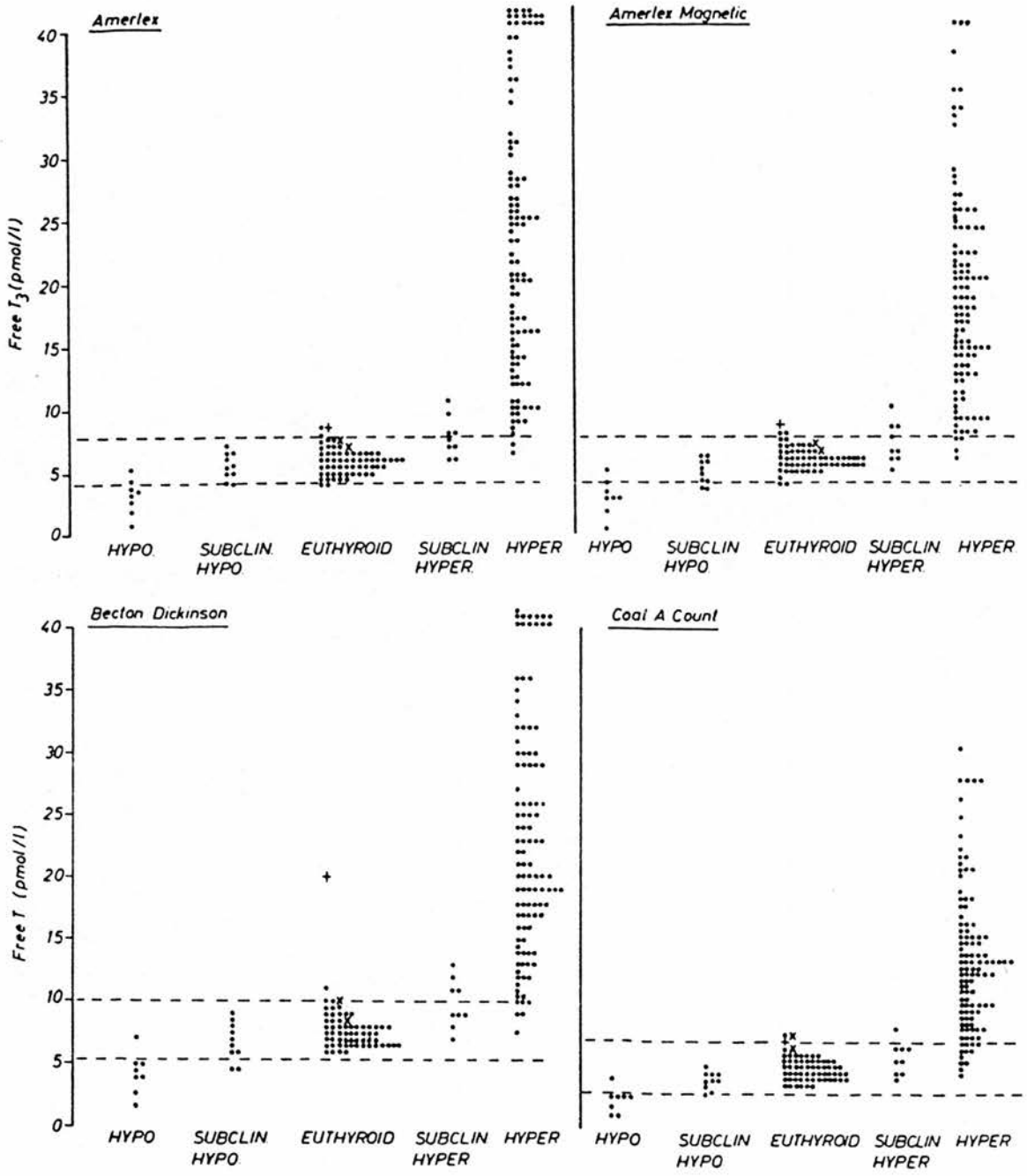


Figure 4.9 Free T_3 Values by Analogue RIA Kits in 200 Patients from a Thyroid Clinic.

Two patients were taking oral contraceptives (x); one patient with high fT_3 had an abnormal high affinity albumin (+).

In contrast, the Coat A Count fT_3 kit produced the only value within reference limits for the euthyroid patient with suspected high-affinity binding to albumin.

As expected, the fT_3 kits did not discriminate effectively those with overt or subclinical hypothyroidism from euthyroid patients. There were differing degrees of overlap between results for hyperthyroid and euthyroid patients with the four kits. The Amerlex method produced the best discrimination; only two hyperthyroid patients were mis-classified. The Coat A Count kit produced the greatest overlap of fT_3 values. Details of the thyroid hormone measurements in these hyperthyroid patients, most of whom had normal total T_3 values, are given in Table 4.6. Free T_3 by dialysis-RIA (when measured) was raised in all of the patients mis-classified by the fT_3 kits.

In patients with subclinical hyperthyroidism, the kits produced similar discrimination and did not appear to resolve this sub-group from euthyroid patients any more clearly than fT_4 measurement (Figure 4.4).

In conclusion, the Amerlex and Amerlex-M fT_3 methods performed best in terms of precision, accuracy and diagnostic effectiveness. The Coat A Count kit was least precise, gave significantly lower fT_3 results and produced the greatest degree of overlap between diagnostic categories. Wilke *et al.* (1984) confirmed these findings

Table 4.6 Hyperthyroid Patients Mis-classified by Analogue RIA Free T_3 Measurement

Patient	Diagnosis	Total T_4 (nmol/l) (70-150)	Total T_3 (nmol/l) (1.1-2.8)	Free T_4 (pmol/l) Dialysis (8-17)	Free T_4 (pmol/l) Amerlex (10-21)	Free T_3 (pmol/l) Dialysis (3.2-6.7)	Free T_3 (pmol/l) Amerlex (4.0-7.8)	Free T_3 (pmol/l) Amerlex-M (4.2-7.8)	Free T_3 (pmol/l) Becton Dickinson (5.3-10.0)	Free T_3 (pmol/l) Coat A Count (3.0-6.9)
1	Toxic multi-nodular goitre	175	2.0	39	36	7.6	6.4	6.4	8.9	5.3
2	Toxic multi-nodular goitre	157	2.5	25	23	7.2	6.8	5.9	7.3	5.0
3	Graves'disease	200	2.5	31	38	7.3	8.8	8.0	9.9	6.6
4	Graves'disease	164	2.9	15	21	7.2	8.0	7.4	8.8	7.5
5	Solitary toxic nodule	159	2.4	-	30	-	8.8	7.6	9.9	7.1
6	Graves'disease	170	3.6	37	37	9.8	10.0	9.2	12.0	5.5
7	Toxic multi-nodular goitre	172	2.3	36	32	9.6	9.3	9.2	10.5	6.8
8	Toxic multi - nodular goitre	157	2.4	28	32	8.0	9.5	8.8	12.3	6.5
9	Graves'disease (on OCP)	164	2.6	34	26	10.2	10.0	8.7	11.6	6.1
10	Graves'disease	168	2.4	26	29	8.3	8.5	8.0	10.3	4.6
11	Toxic multi-nodular goitre	137	2.9	-	32	-	10.6	8.9	12.0	6.3

Results within reference limits are underlined.

and demonstrated some TBG-dependence of Coat A Count results which may explain the decreased sensitivity of this test. This kit has since been modified by the manufacturer.

4.4 FREE T_4 AND TSH BY DUAL-ANALYTE RIA

The SimulTRAC fT_4/TSH dual RIA kit was developed to combine the diagnostic strengths of TSH and fT_4 measurements. Free T_4 is measured using a ^{57}Co-labelled T_4 analogue which is claimed not to bind significantly to the natural binding proteins in serum. A different label (^{125}I-TSH) is used in the estimation of TSH and, therefore, the two measurements can be performed in the same tube provided a gamma counter is used which is capable of distinguishing the different emission energies of the two radioisotopes.

4.4.1 The SimulTRAC Free T_4/TSH RIA Protocol

The manufacturer's protocol for the SimulTRAC fT_4/TSH RIA is shown in Figure 4.10. The standards provided were calibrated against the 68/38 IRP for TSH and an equilibrium dialysis procedure for fT_4. Using the high sensitivity option for the assay, the top standards used had values of 15 mU/l for TSH and 70 pmol/l for fT_4.

Standards A-F and samples (200 µl) were incubated in duplicate with rabbit dual antibody (100 µl) for 2.5 h at 37°C then for 1.5 h more after adding 100 µl of dual tracer. The batch size was limited to 20 patient samples

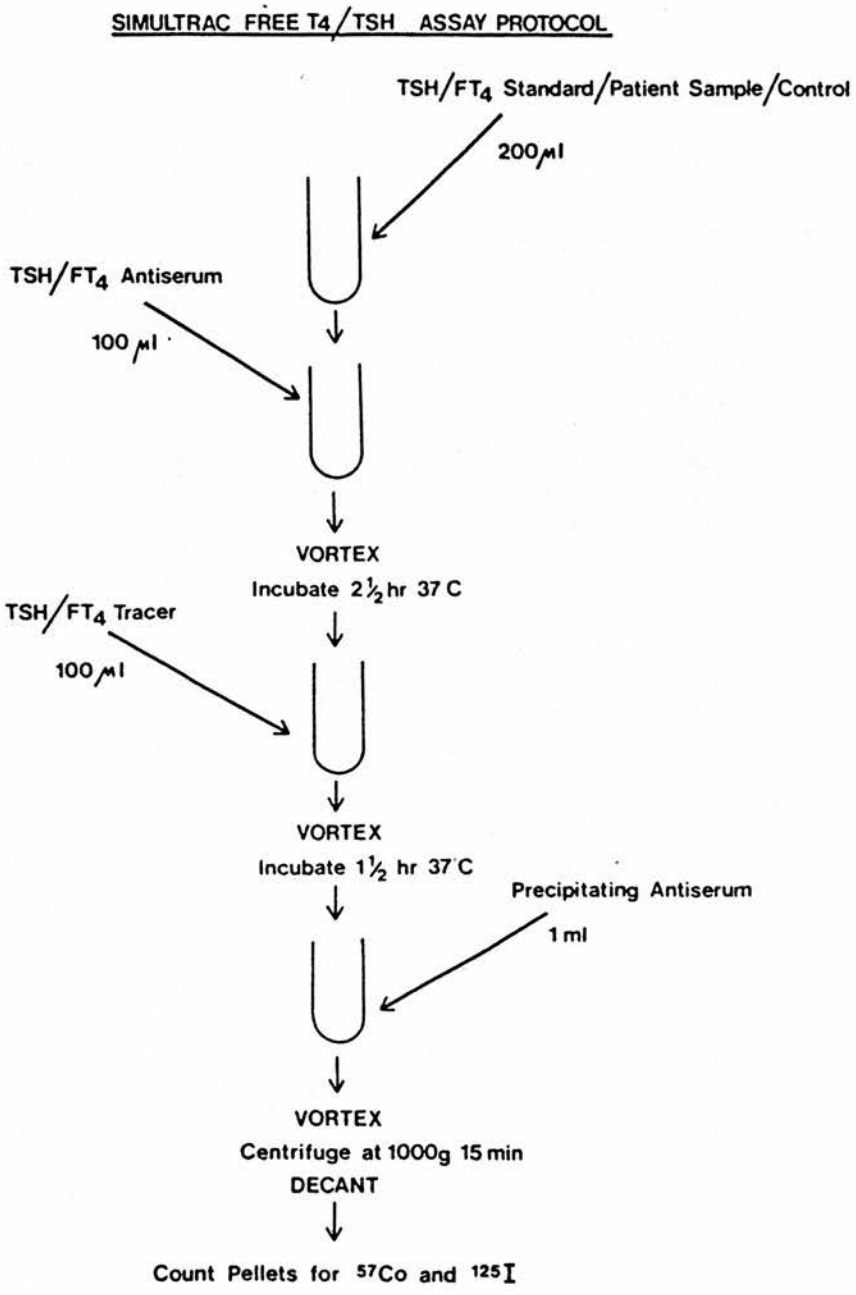


Figure 4.10 The SimulTRAC Free T4/TSH RIA Protocol.

as recommended by the manufacturer. Free ligand was separated from bound by adding goat anti-rabbit antiserum plus PEG to accelerate the precipitation and then centrifuging. Radioactivity from the two isotopes was counted simultaneously in the LKB Wallac gamma counter. The percentage spill-over in each channel, assessed using pure ^{57}Co and ^{125}I counting standards, was less than 2% for each assay. Pellets were counted for 60-90 sec to achieve the recommended total counts for each tracer (20,000 for ^{57}Co and 25,000 for ^{125}I).

4.4.2 The Precision and Bias of the Dual-Analyte Assay

The mean within-assay precision profiles (Figure 4.11) were calculated from results for duplicate sample analyses from 11 SimulTRAC assays. The fT_4 assay had excellent precision over a wide concentration range, whereas the TSH assay was less precise with a mean CV of 10% at 1.0 mU/l.

The between-assay precision data for in-house serum pools and Lyphochek human control serum (Bio-Rad Richmond, California, U.S.A.; lot no. 07300) over 11 assays are shown in Table 4.7. In addition, the between-assay CVs from 11 RACs (range 7.4-27.5 pmol/l for fT_4, 0.3 - 58 mU/l for TSH) were 6.8% for fT_4 and 11.9% for TSH.

The SimulTRAC fT_4 assay had better precision than the Becton Dickinson coated-tube fT_4 assay and compared favourably with the Amerlex-M fT_4 method (Table 4.4),

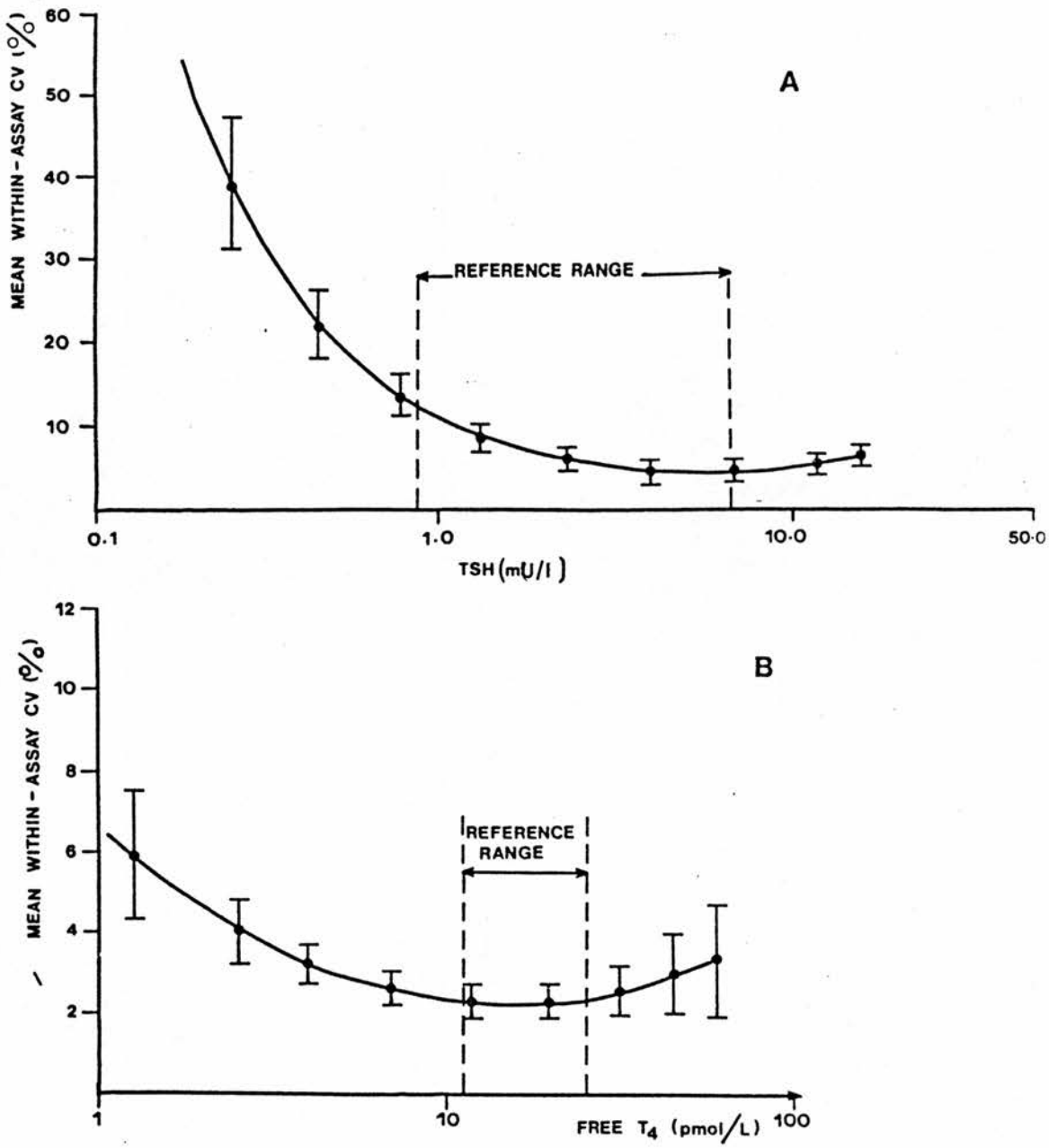


Figure 4.11 Within-assay Precision Profiles (Mean±SD, n=11) for the SimulTRAC TSH (A) and Free T_4 (B) RIA.

Table 4.7 Between-assay Precision of the SimulTRAC Free T_4/TSH Assay

FT_4, pmol/l	Lyphochek			Pooled sera	
	I	II	III	1	2
*Mean	3.2	9.7	38.9	15.6	41.0
Target	3.3	9.3	37.3	15.0	40.0
Bias, %	-3	+4	+ 4	+ 4	+ 2
SD	0.2	0.4	0.9	1.0	2.6
CV, %	5.8	4.2	2.3	6.2	6.4
TSH, mU/l	I	II	III	a	b c
*Mean	1.5	10.9	30.2	3.0	9.0 42.9
Target	1.5	11.2	23.2	2.8	7.5 44.8
Bias, %	0	- 3	+30	+7	+20 - 4
SD	0.1	1.0	2.7	0.2	0.5 2.2
CV, %	10.2	9.4	8.8	7.7	6.1 5.2

*n = 11

previously considered to be the analogue fT_4 method of choice. The SimulTRAC TSH assay had a slightly wider working range (1.0-15 mU/l) than the in-house TSH RIA (1.0-10 mU/l) shown in Figure 2.2, and the between-assay precision from the quality control pools compared favourably with that of the in-house TSH assay (Table 2.3). The CVs for Lyphochek controls and RACs however,

were slightly higher than those seen for pools and RACs using the in-house assay. The gamma counter window settings used to minimise spill-over tended to favour the counting of ^{57}Co compared to ^{125}I, such that the counting rate in total count tubes for ^{57}Co (fT_4) was 40,000 cpm and that for ^{125}I (TSH) was 25,000 cpm. Since the SimulTRAC TSH assay operates with a B_o of 30%, improved precision may have resulted had the recommended counting time been exceeded.

The average bias for the Lyphochek controls was calculated from the quoted values. For the in-house pools, bias was calculated from target values established by the Amerlex-M fT_4 assay for pools 1 and 2 and by the in-house TSH RIA for pools a-c (Table 4.7). The Lyphochek III and pool c were diluted five-fold in zero standard before measuring TSH. This pre-dilution may have contributed to the variable bias figures obtained for the SimulTRAC TSH assay at the higher TSH concentrations.

4.4.3 Detection Limit and Recovery of the TSH Assay

The detection limit, determined conventionally from the 97.5% (2.5 SD) confidence limits of 20 replicate estimations of the zero standard (Rodbard, 1978) was 0.17 mU/l. Since patient samples are not assayed in replicates of 20, a more meaningful 2.5 SD estimate of 0.25 mU/l was obtained from the mean intra-assay precision profile by extrapolation of the asymptote of the SD pro-

file to the SD value at zero dose (John & Jones, 1984; Ekins, 1983b). This was lower than that of the in-house RIA and not far removed from detection limits described for TSH by IRMA in the following section.

The analytical recovery of the SimulTRAC TSH assay was calculated from pooled euthyroid serum with 1, 4 and 20 mU/l of added TSH IRP (final concentration) measured in 3 assays. The mean recoveries for these additions were 113%, 120% and 97%,respectively.

4.4.4 Correlation with other Methods

Regression analysis of fT_4 results obtained by SimulTRAC (y) and the Amerlex-M fT_4 method (x) for 32 patients' samples analysed in the routine diagnostic laboratory because of suspected alterations in protein binding (range 4-35 pmol/l) gave the equation: $y = 1.13x-1.7$ pmol/l. TSH values in 116 patient samples from the thyroid clinic did not have a normal distribution. SimulTRAC TSH values (y') were therefore compared with the in-house TSH RIA (x') in only 50 samples where TSH was detectable by both methods but less than 8 mU/l. This gave the regression equation: $y' = 1.08x' - 0.44$. These correlations were significantly different ($p<0.001$) from $y=x$ for the x slope and the y' intercept. TSH values in 11 patients with TSH >8.0 mU/l were significantly lower ($p<0.05$; paired *t*-test) by SimulTRAC (mean 60.9±40.2 mU/l) compared to the in-house assay (mean

103.2±92.0 mU/l). However, this difference was not sufficient to affect the patient categorisation.

4.4.5 Diagnostic Accuracy in Patient Samples

The diagnostic performance of the SimulTRAC assay was assessed in 116 consecutive patients attending the thyroid clinic. These were different patients from those in other sections of this chapter but they were categorised in the same way. Seven of 40 euthyroid patients in this group were taking an OCP.

The 95% confidence intervals calculated from the 40 euthyroid patients were 11.1-24.1 pmol/l for fT_4 and 0.6-5.7 (mean 1.8) mU/l for TSH, after logarithmic transformation of the TSH data. These intervals were slightly lower than those derived by the manufacturer from results for 146 individuals: fT_4, 11.4-25.3 pmol/l; TSH, 0.9-6.7 mU/l. Use of the manufacturer's reference range for TSH minimised the overlap between the different categories of patients (Figure 4.12).

All hyperthyroid patients (overt and subclinical) had TSH values <0.9 mU/l and all hypothyroid patients (overt and subclinical) had TSH values >6.7 mU/l. With these reference limits, results overlapped for only two euthyroid patients (5%) who had low concentrations of TSH (0.6 and 0.8 mU/l). However, this degree of overlap in basal SimulTRAC TSH would militate against the use of this TSH method as a predictor of the TRH test response, as discussed further in Section 4.5.

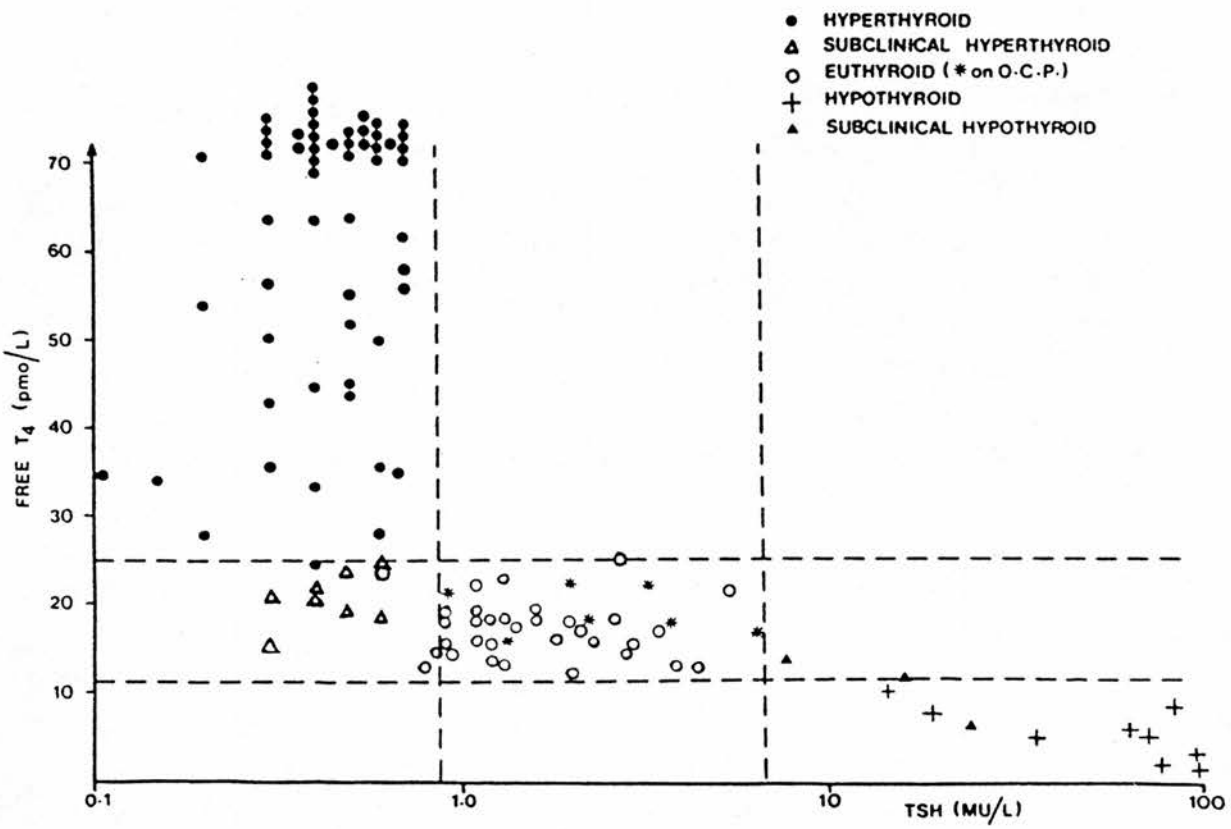


Figure 4.12 SimulTRAC Free T4/TSH RIA Results in 116 Patients from a Thyroid Clinic.

Dashed lines indicate reference ranges: fT4 horizontal, TSH vertical.

All patients with overt hyperthyroidism (n=56) had fT_4 values greater than 24.1 pmol/l, and all those with overt hypothyroidism (n=9) had fT_4 values below 11.1 pmol/l. All but two patients with subclinical disease (n=11) had fT_4 concentrations within the reference interval: one patient with subclinical hyperthyroidism had a fT_4 value of 24.8 pmol/l, and one with subclinical hypothyroidism had a low fT_4 result (6.9 pmol/l), Figure 4.12. All euthyroid patients whose total T_4 concentrations were increased due to the OCP, had normal SimulTRAC results.

All patients with overt hyperthyroidism were distinguished from euthyroid patients when TSH and fT_4 results were combined. Therefore, compared to our standard strategy for investigating new patients referred to a thyroid clinic, the SimulTRAC assay produced similar categorisation in 112/116 (96.5%) of patients. This was achieved using individual reference ranges for fT_4 and TSH without recourse to more complex linear discriminant analysis which has been applied in the past to combinations of older thyroid function tests (Barnett et al., 1973).

4.5 MEASUREMENTS OF TSH BY IMMUNORADIOMETRIC ASSAY

Two high sensitivity IRMA methods for TSH were evaluated. Both employ a soluble ^{125}I-labelled mouse monoclonal antibody and a second mouse monoclonal coupled to solid-phase which is directed against a different epitope of TSH. In these sandwich assays, the radiolabel

bound to the solid-phase is directly proportional to the TSH concentration of the sample (Figure 1.4).

4.5.1 The Boots-Celltech Sucrosep TSH IRMA

The TSH standards (0-200 mU/l) were supplied in a bovine serum-based matrix and calibrated against the 68/38 IRP and the 80/558 IRP. Standards and samples (100 µl) were pipetted manually in duplicate into assay tubes with fresh pipette tips to avoid carry-over problems. A repeat-dispenser (Hamilton) was used to add the ^{125}I-anti-TSH (100 µl) and, after mixing, the tubes were incubated at room temperature for 2 h. Anti-TSH, covalently coupled to Sephacryl particles in suspension (100 µl), was then dispensed to all tubes and the incubates shaken on an orbital agitator at 300-350 rpm for 1 h at room temperature. Tubes were allowed to stand for 5 min after the addition of pre-wash buffer (1 ml) and then the "Sucrosep" reagent was layered beneath the incubates using the manufacturer's 20-probe sucroseparator. As described by Wright and Hunter (1983), excess radiolabel was washed from the solid-phase by gravity sedimentation through 10% sucrose (15 min) followed by aspiration to waste of the sucrose layer containing the unbound ^{125}I-antibody (Figure 4.13). This sucrose separation was repeated once to achieve the required low (<0.1%) NSB values. The bound radiolabel was then counted in the Wallac gamma counter with the counting windows set to detect only the major

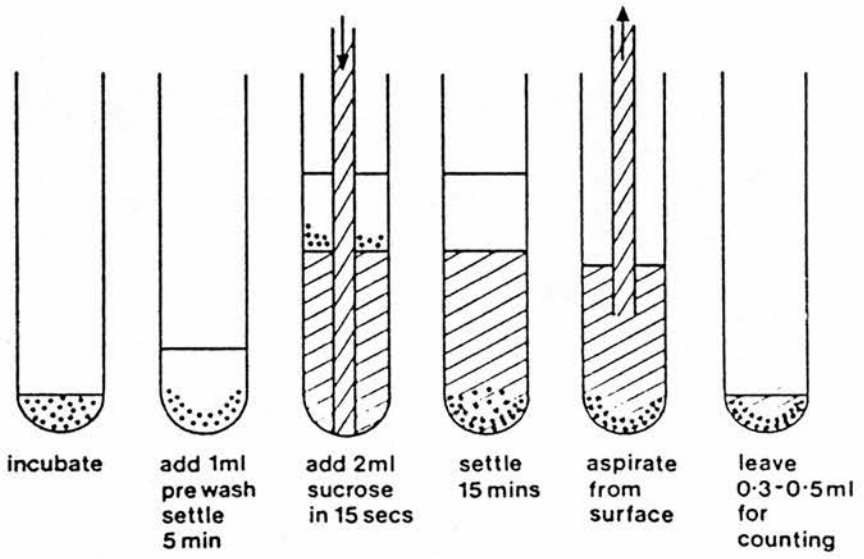


Figure 4.13 The Sucrose Layering Technique for the Separation of Free and Bound Fractions of Particulate Solid-phase Immunoassays.

peak of the iodine spectrum. The Edinburgh Immunoassay package was used for curve-fitting and data interpolation.

In this thesis, half of the assays by this method were performed by staff of the Immunoassay Section of this department (principally Chapter 4) and half by myself.

4.5.2 The Amerwell TSH IRMA

Each Amerwell TSH Kit contains a microtitre plate consisting of 8 x 12 microwell strips coated with anti-TSH, 8 human serum standards calibrated against the 2nd IRP (80/538) and soluble ^{125}I-anti-TSH. Radiolabel (50 µl) was dispensed to each well followed by standards, samples and controls (200 µl; Finn pipette) in duplicate (20 min). The plates were incubated for 2 h at room temperature on an Amerwell shaker and then washed four times with borate buffer (supplied by the manufacturer) using the Amerwell 12-pronged washer/aspirator (Figure 4.14). The individual wells were counted in the NE1600 multiwell gamma counter for 120 s. Care was taken to ensure that background counts in each counter-well and counting cassettes were always <1 per second. Curve-plotting and data interpolation were performed using the WHO immunoassay package (single binding-site model). The lowest three standards and corresponding ^{125}I count rates were re-plotted on linear-linear graph paper if there was evidence of misfitting of the low (0.2 mU/l) standard for interpolation of low TSH concentrations. In general,

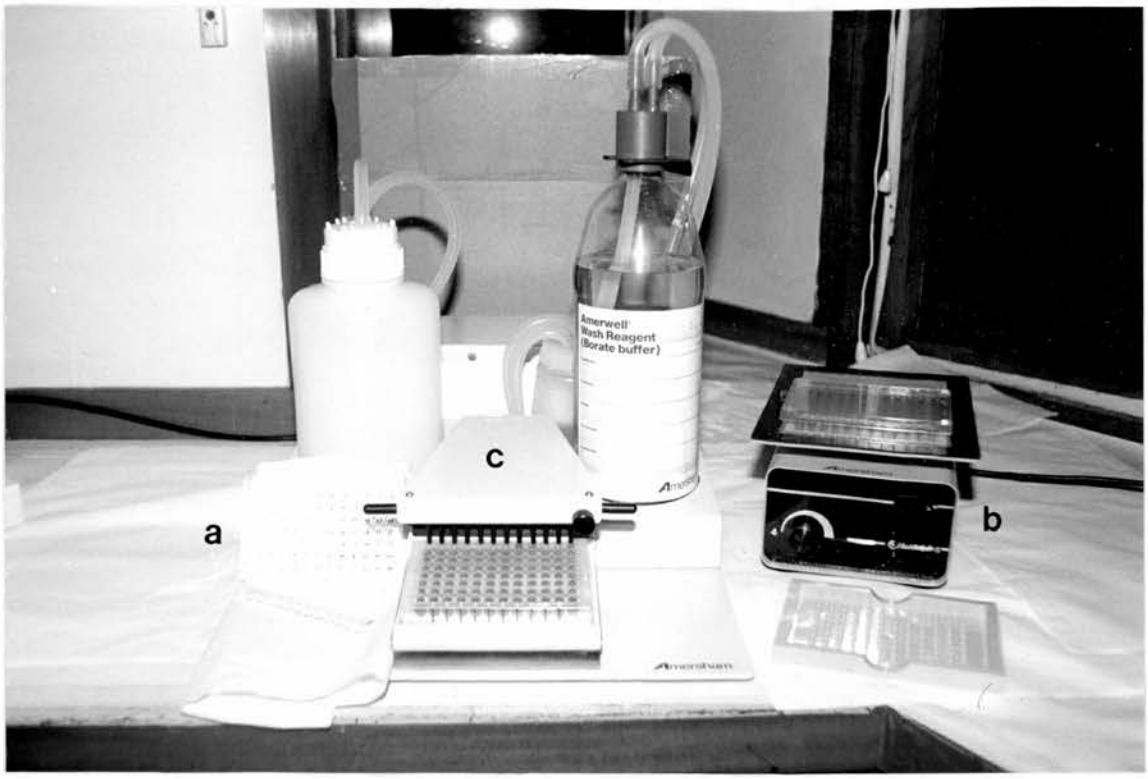


Figure 4.14 Equipment Used in the Amerwell TSH IRMA.

(a) Microtitre plate with numbered wells.
(b) Amerwell orbital shaker.
(c) Amerwell washer/aspirator.

there was good agreement between the data interpolation and precision profiles generated by the Edinburgh (x) and WHO (y) immunoassay packages provided the 0.2 mU/l standard was correctly fitted by the latter (y = 1.02x-0.06, n=51 from 3 assays; TSH range 0.14-35 mU/l).

4.5.3 Comparison of IRMA and RIA Protocols for TSH

The requirement to reduce misclassification errors and the NSB is important for immunometric assays if their sensitivity potential is to be achieved (Jackson et al., 1983); with RIA, the antibody affinity is the main determinant of assay sensitivity. Wash procedures, therefore, form a more prominent part of the IRMA compared to the RIA. The main differences in the manipulations and the total assay time of the two IRMA methods described here arise because of the different solid supports of the capture antibody and the wash steps adopted to reduce the NSB values (Table 4.8). The washing of microtitre plates was more convenient, rapid and could be readily automated in contrast to the more time-consuming and less robust sucrose separation.

Since the IRMA methods use excess reagents, the reaction kinetics are favourable for the assay to be completed in a relatively short incubation time. The in-house assay, by comparison, requires a prolonged incubation (48 h) with delayed tracer addition in order to achieve good sensitivity (Section 2.2.4). The protocol for the Becton Dickinson ^{125}I-TSH RIA kit (Table 4.8)

Table 4.8 Comparison of TSH Assays

	Amerwell IRMA	Boots-Celltech IRMA	In-house RIA	Becton Dickinson RIA
Tubes or wells/kit	96	120	-	100
Sample vol (µl)	200	100	100	200
Pipetting steps	2	3	3	3
Incubation time (h)	2	3	48	4
Separation	4 washes	2 pre-washes 2 sucrose layers	Double-antibody precipitation and filtration	Double-antibody with PEG and centrifugation
Separation time	5 min	40 min	18 h	20 min
Total assay time	2½ h	4 h	4 days	5 h
Sensitivity (mU/l)	0.07	0.07	0.58	0.23
*Detection limit (mU/l)	0.09	0.10	0.41	0.30
*Working range (mU/l)	0.3-200	0.6-240	0.9-10	1.2-30

(Sensitivity, detection limit and working range are defined in the text).
*Mean for 10 assays.

demonstrates however, that same-day results can be produced by RIA. This RIA employed delayed tracer addition and PEG-accelerated double antibody precipitation, the procedure being identical to that previously described for the SimulTRAC RIA (Figure 4.10) but with use of the NE1600 multiwell gamma counter for counting the label.

4.5.4 Assay Sensitivity and the Working Range

For each TSH method the assay sensitivity was calculated as the TSH concentration giving a signal 2.5SD from the mean of 20 replicates of the zero standard (Table 4.8). Using this definition of sensitivity (Rodbard, 1978), a nine-fold and three-fold increase in sensitivity were achieved with the IRMA methods compared to the in-house and the Becton Dickinson RIA methods, respectively. These values were comparable to the detection limit calculated for each method from the mean within-assay precision profiles of patient samples analysed in duplicate (Figure 4.15) which showed that the IRMA methods had three to four-fold lower detection limits compared to RIA (Table 4.8). This raised the possibility of distinguishing low from normal TSH concentrations in serum. The IRMA methods also had better within-assay precision and much wider working ranges (CV <10%) compared to RIA which permitted the analysis of samples containing high levels of TSH without prior dilution.

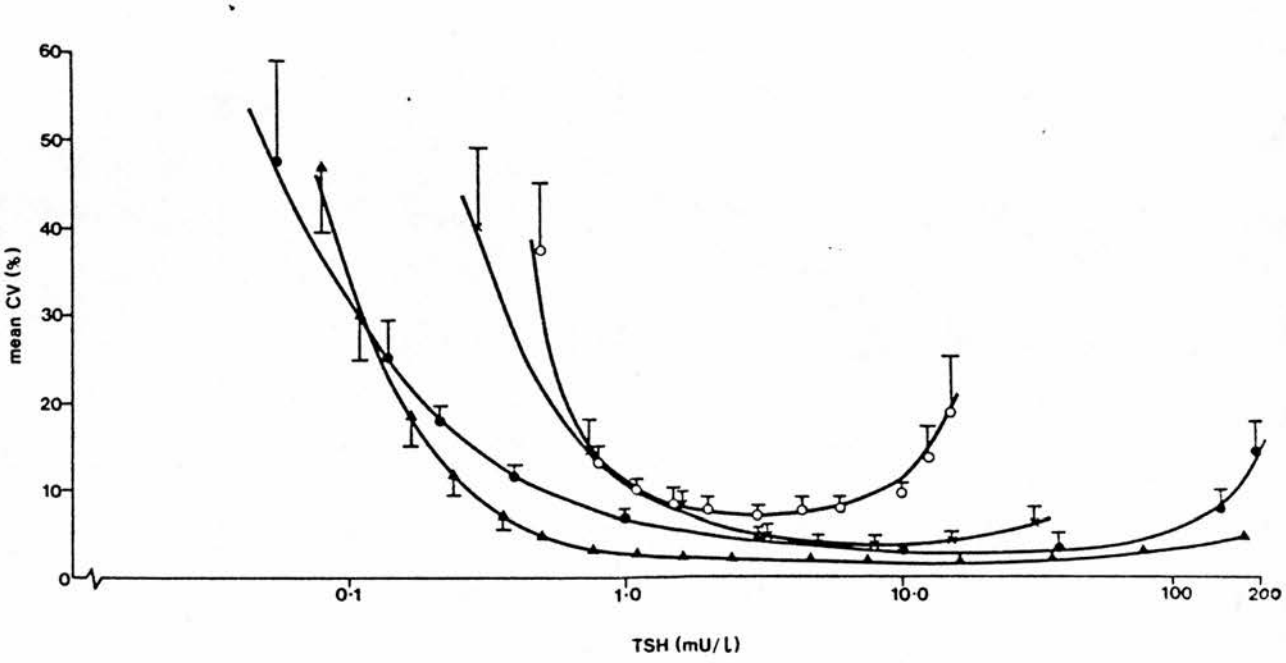


Figure 4.15 Within-assay Precision Profiles (Mean±SD, n=12) for TSH Methods.

In-house RIA (o——o),
Becton Dickinson RIA (x——x),
Boots-Celltech IRMA (●——●),
Amerwell IRMA (▲——▲).

4.5.5 The Between-assay Precision of the IRMA Methods

Data for the between-assay precision from at least 10 assays is given in Table 4.9. In-house pools and control sera supplied by the manufacturers were used in the evaluation. Data for the in-house RIA are shown in Table 2.3. A low pool (pool a) was prepared from the serum of patients with TSH levels <0.9 mU/l by RIA to assess the precision of the Amerwell IRMA at the lower reference limit. Bias values were calculated from in-house RIA targets for the pools or the manufacturers' quoted values. The Amerwell IRMA appeared to be more precise at low TSH values than the Boots-Celltech assay but produced a marked negative bias with respect to the target values for in-house pools.

Table 4.9 Between-assay Precision of the TSH Methods

	Boots-Celltech IRMA				Amerwell IRMA			
	Mean (mU/l)	Bias+ (%)	SD (mU/l)	CV (%)	Mean (mU/l)	Bias+ (%)	SD (mU/l)	CV (%)
	In-house pools				In-house pools			
a	-	-	-	-	0.3	-	0.03	11.1
1*	2.0	-13	0.25	12.6	1.7	-26	0.09	5.5
2	8.7	+16	0.56	6.4	5.7	-24	0.26	4.6
3	21.1	-10	1.15	5.5	-	-	-	-
4	47.3	+ 4	3.29	7.0	30.7	-32	3.29	10.7
	Boots-Celltech Controls				Amerwell Controls			
1	0.3	-17	0.05	14.2	1.3	0	0.06	4.7
2	2.2	+ 4	0.09	4.0	3.9	- 5	0.16	4.1
3	5.8	- 1	0.23	4.0	19.9	- 5	1.16	6.0
4	17.8	- 5	0.89	5.0				
5	46.4	- 4	2.29	4.9				

+See text.
*New in-house pool compared with Table 2.3

In both IRMA methods a slight downwards drift in values (Boots-Celltech -6.5%; Amerwell -4.0%) was observed for samples analysed at the beginning and the end of an assay. This was not found to be of any major significance in patient categorisation.

4.5.6 Correlations Between TSH Methods

Since the results from patients in the study were not normally distributed, regression analysis was performed (a) after logarithmic transformation and (b) for TSH values less than 8 mU/l only. After logarithmic transformation, all of the methods correlated well with the in-house RIA ($r > 0.96$; $n = 80$). There was also a good correlation between the two TSH IRMA methods (Figure 4.16A). However, this transformation obscured the true relationship between TSH values by different methods (Figure 4.16B and Table 4.10).

Table 4.10 Correlation of TSH Values <8 mU/l ($y = mx + c$)

y	x	r	m(SE)	c(SE)
IRMA (BC)	RIA (I)	0.905	1.08(0.07)	-0.38*(0.16)
IRMA (AM)	RIA (I)	0.877	0.62*(0.07)	0.15 (0.17)
RIA (BD)	RIA (I)	0.922	0.99 (0.05)	0.54*(0.14)
IRMA (AM)	IRMA (BC)	0.892	0.60*(0.04)	0.34*(0.10)

BC=Boots-Celltech; I=In-house; AM=Amerwell; BD= Becton Dickinson. *Significant differences from $y = x$.

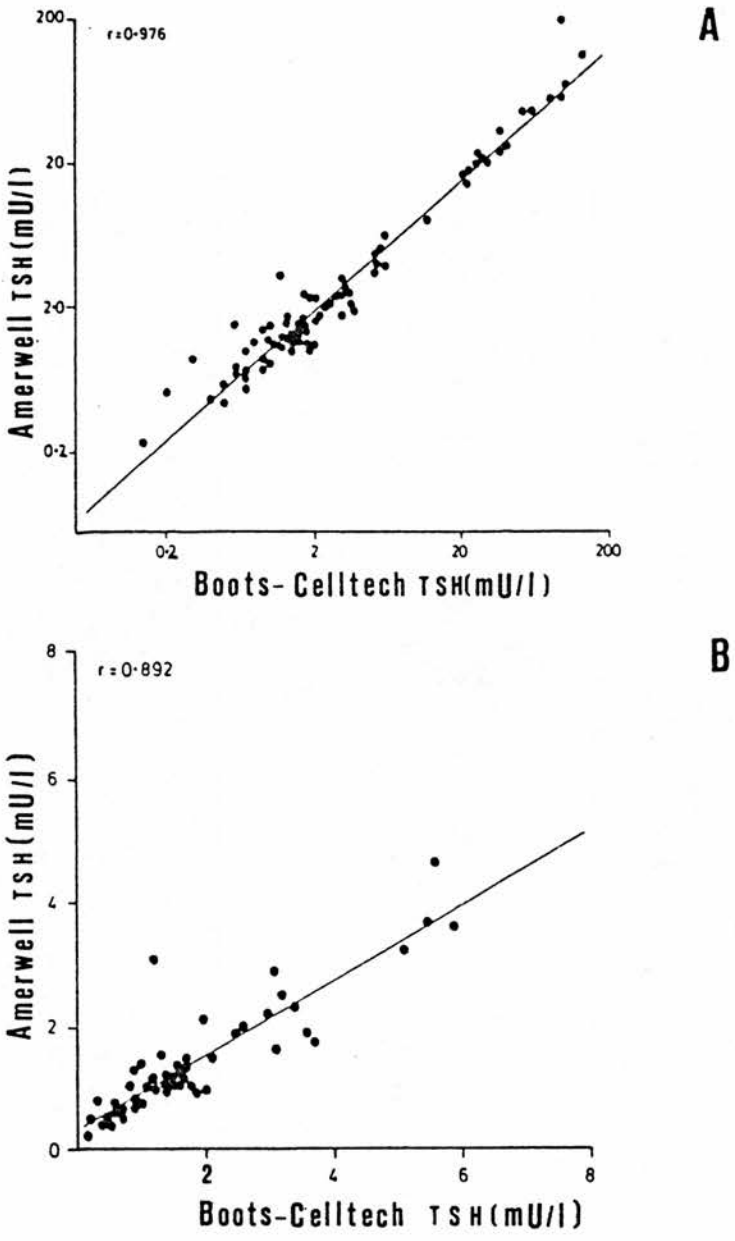


Figure 4.16 Correlations of TSH Values by Two IRMA Methods

(A) Logarithmic transformation of all data points and (B) linear regression for TSH values <8 mU/l.

Although there was good agreement between values measured by the Boots-Celltech IRMA and the RIA methods, values by the Amerwell IRMA were approximately 40% lower. This discrepancy was not entirely due to poor analytical recovery of the Amerwell method since, the mean recovery of TSH when 0.5-20 mU/l of IRP 80/558 was added to three euthyroid serum pools was 94% (range 84-101, n=13) and when 0.5-1.5 mU/l IRP was added to T_3-suppressed serum, 100% and 85% TSH was recovered, respectively. There was also no evidence of nonlinearity for two-fold and four-fold dilutions of patients' sera, using sera collected from thyrotoxic patients as diluent.

4.5.7 Categorisation of Patients using TSH IRMA

The TSH results by IRMA are illustrated in Figures 4.17 and 4.18 for the 200 consecutive patients. The absolute ranges of values for the 63 euthyroid patients were 0.20-5.9 mU/l (Boots-Celltech) and 0.36-4.5 mU/l (Amerwell); these were used as reference ranges in this section.

All hypothyroid patients (overt and subclinical) had raised basal TSH concentrations by both assays. None of the overt hyperthyroid patients had TSH detectable in serum by either method. In the 15 patients with subclinical hyperthyroidism all but one had undetectable TSH by Boots-Celltech IRMA, whereas by Amerwell assay, TSH was detectable but low in 5 patients, 4 of whom had multinodular goitres. Results for the TRH stimulation tests in these patients are shown in Table 4.11 (Patients 1-4).

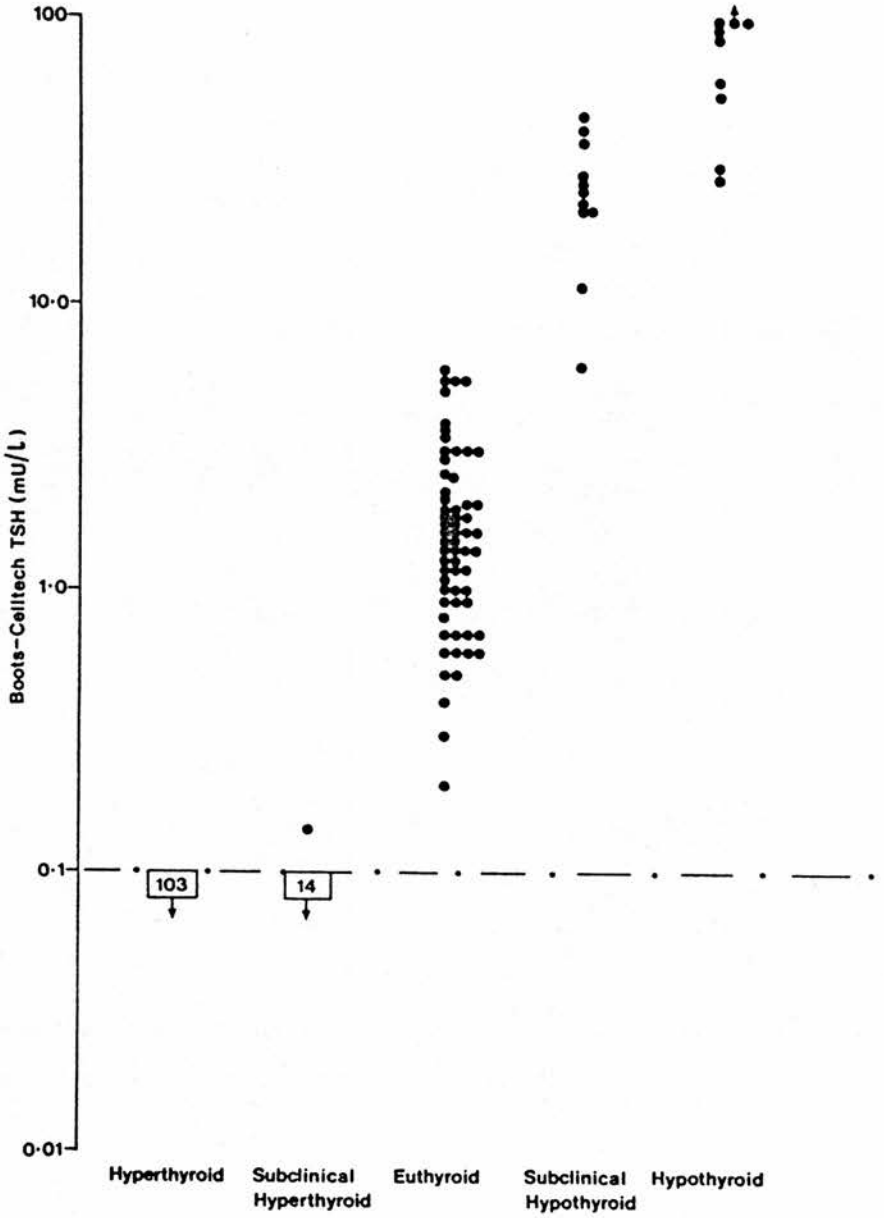


Figure 4.17 Values for Boots-Celltech TSH IRMA in 200 Patients from a Thyroid Clinic.

The mean minimum detection limit is indicated (—·—).

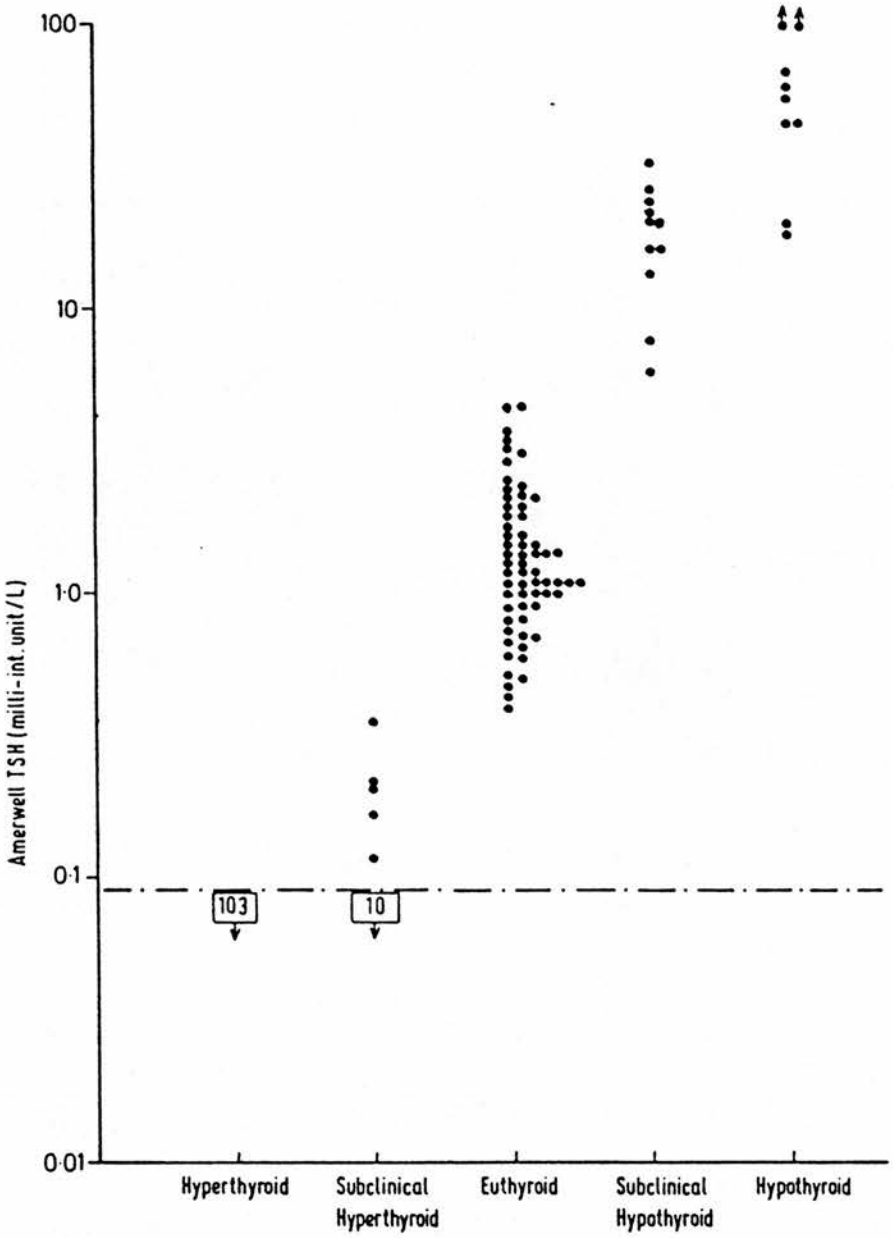


Figure 4.18 Values for Amerwell TSH IRMA in 200 Patients from a Thyroid Clinic.

The mean minimum detection limit is indicated (- • -).

Table 4.11 Basal TSH and the Response After TRH in Five Patients with Subclinical Hyperthyroidism

Patient	TSH (mU/l)					
	Boots-Celltech IRMA		Amerwell IRMA		In-house RIA	
	0 min	20 min	0 min	20 min	0 min	20 min
1	<0.13	1.30	0.35	ND	<1.0	1.2
2	<0.10	1.30	0.22	ND	<0.9	1.8
3	<0.15	0.26	0.17	ND	<1.1	<1.1
4	<0.10	0.58	0.12	ND	<0.7	1.0
5	0.14	1.5	0.21	ND	1.0	1.9

ND = Not determined.

By Boots-Celltech IRMA and the in-house RIA, TSH was undetectable in the basal sample. An increase in TSH greater than 1.0 mU/l after TRH was measured in sera from patients 1 and 2 using the Boots-Celltech IRMA and a smaller but discernible rise was measured in sera from patients 3 and 4 who had the lower basal Amerwell TSH concentrations. These results are consistent with incomplete suppression of thyrotroph function. One patient (Patient 5) had a low but detectable TSH concentration by both IRMA methods. She was clinically euthyroid, had normal serum total T_4 and T_3 and a marginally subnormal response to TRH as measured by RIA but which was greater than 1.0 mU/l by IRMA. She presented with a solitary thyroid nodule which regressed spontaneously after three months, suggesting a diagnosis of haemorrhage into a cyst.

The fact that TSH concentrations were undetectable by Boots-Celltech IRMA but detected in the Amerwell IRMA in four patients with subclinical hyperthyroidism, may be a feature of the particular monoclonal antibody combination used or different matrix effects in the two kits. Wiersinga et al. (1986) have also found some patients with subclinical hyperthyroidism who have little response to TRH but a low basal TSH level detected by immunofluorometric assay. It is clear, however, from the results in this study that TSH IRMA distinguished effectively euthyroid from hyperthyroid patients. This was clearly not the case for TSH measured by RIA (Figure 4.19) where some euthyroid patients (4/63) had values less than the reporting limits (working range) of the RIA and some hyperthyroid patients (9/103 overt; 3/15 subclinical) had values >0.9 mU/l. In contrast to IRMA, measurements of TSH by this RIA could not therefore be used to predict the result of a TRH test.

4.6 AN EXTENDED CLINICAL STUDY FOR THE ASSESSMENT AND COMPARISON OF THYROID FUNCTION TESTS

Measurements of free thyroid hormones and TSH IRMA were performed in the complete series of 285 patients (Table 4.2) using Amerlex fT_4 and fT_3 kits and the Boots-Celltech Sucrosep IRMA.

4.6.1 Basal TSH IRMA Concentrations

The concentrations of TSH in the 97 patients categorised as euthyroid (Table 4.3) were all detectable and

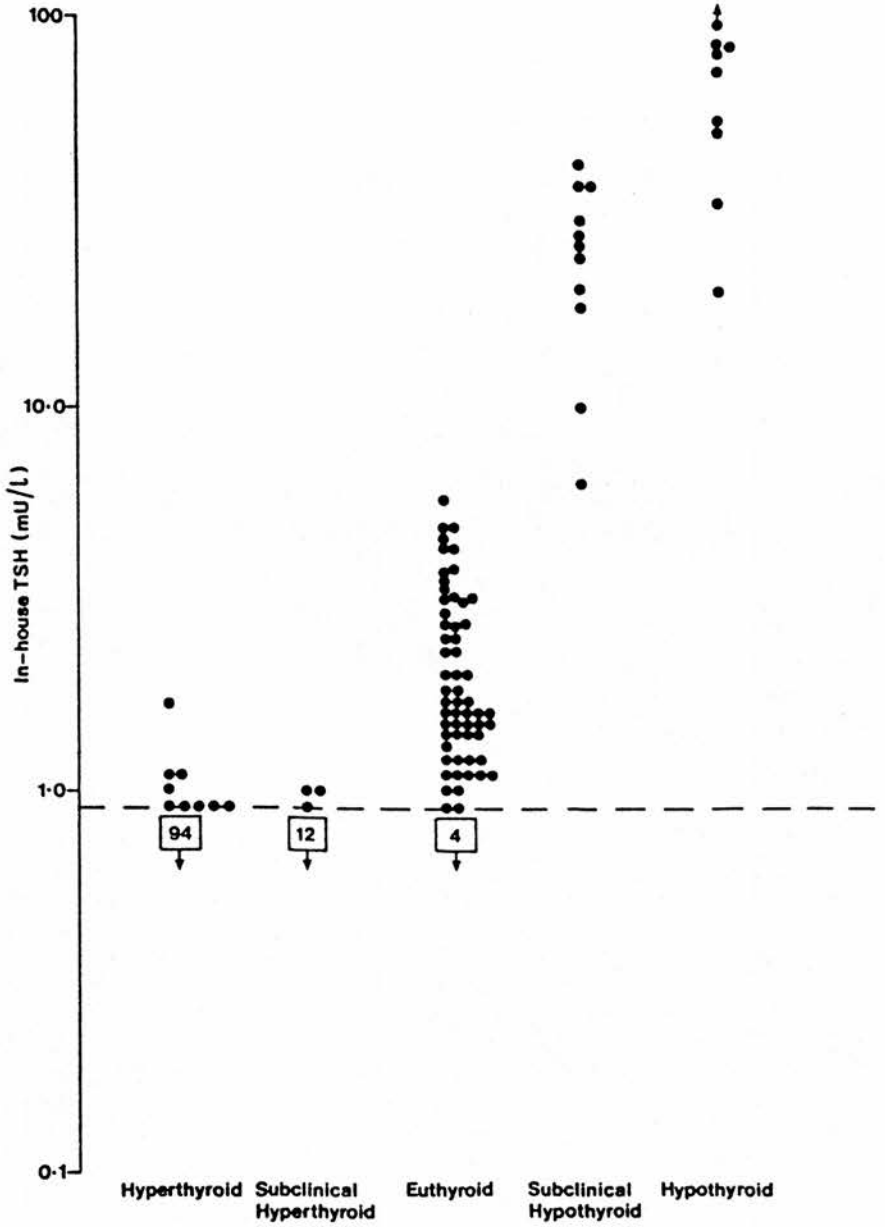


Figure 4.19 Basal TSH by In-house RIA in 200 Patients from a Thyroid Clinic.

The lower reporting limit is indicated (- - -).

ranged from 0.14 to 5.9 mU/l (mean 1.9 mU/l), Figure 4.20. This absolute range provided a good separation of euthyroid patients from those with thyroid disease (overt and subclinical) and was, therefore, used as the reference interval in all further studies.

All but one patient with an absent TSH response to TRH by RIA had an undetectable basal TSH by IRMA. This patient whose TRH test was marginally abnormal appeared to have had a transient episode of subclinical hyperthyroidism with a palpable thyroid nodule which subsequently regressed, as discussed previously (Section 4.5.7).

4.6.2 Free T_4 Compared with Total T_4 Concentrations

The Amerlex fT_4 results are shown in Figure 4.21 and compared with total T_4 values. The reference limits shown for total T_4 were those used in the patient categorisation whereas those for fT_4 (10.0-22.5 pmol/l) represent the mean±2SD for this euthyroid group, excluding the one patient with abnormal albumin binding.

In patients with overt hyperthyroidism, fT_4 concentrations were more markedly elevated than total T_4: 95% of hyperthyroid patients had fT_4 values greater than 4 SD above the mean euthyroid value compared to only 57% for total T_4. Free T_4 concentrations were raised in 9 of the 11 patients with T_3-thyrotoxicosis. In those with subclinical hyperthyroidism, if categorisation had rested on

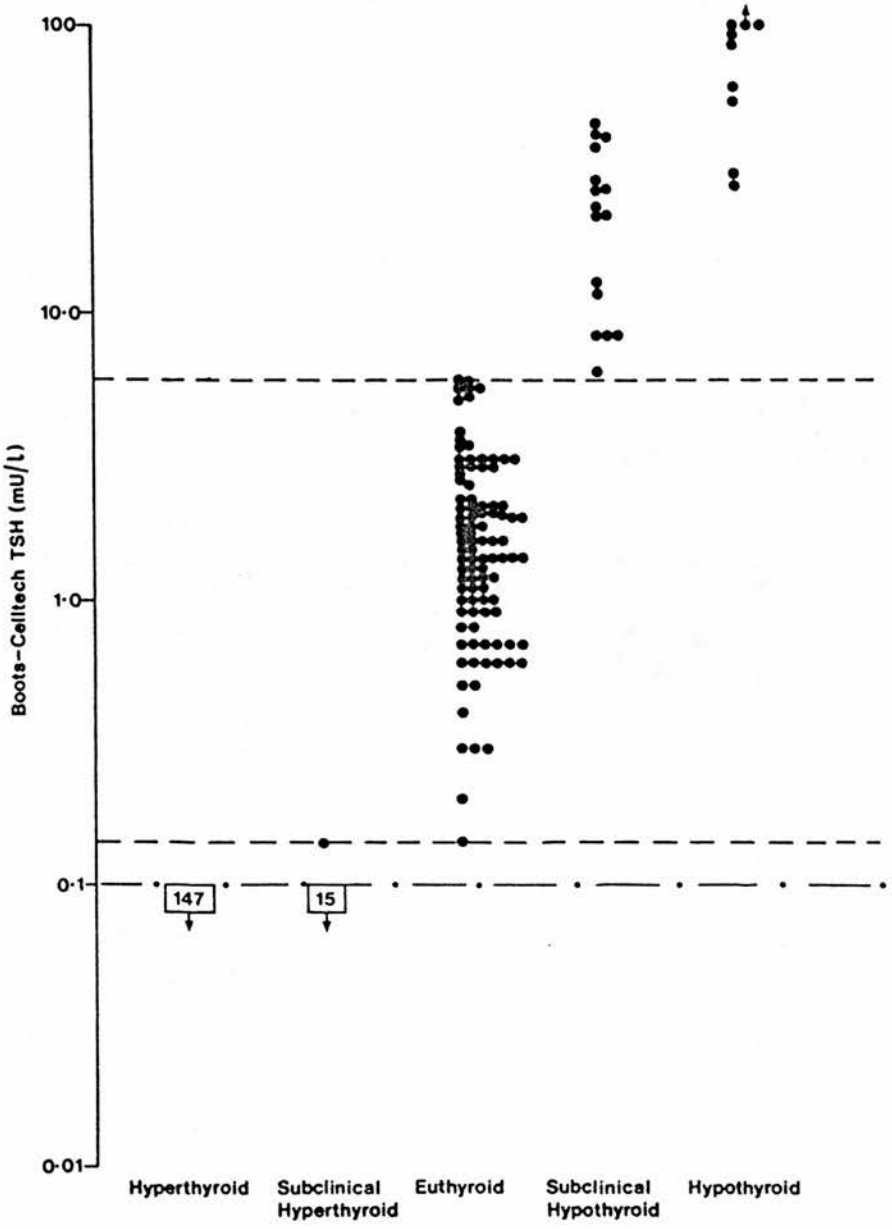


Figure 4.20 Boots-Celltech TSH IRMA Values in Serum from 285 Patients from a Thyroid Clinic.

Reference limits (- - -)
Minimum detection limit (—·—).

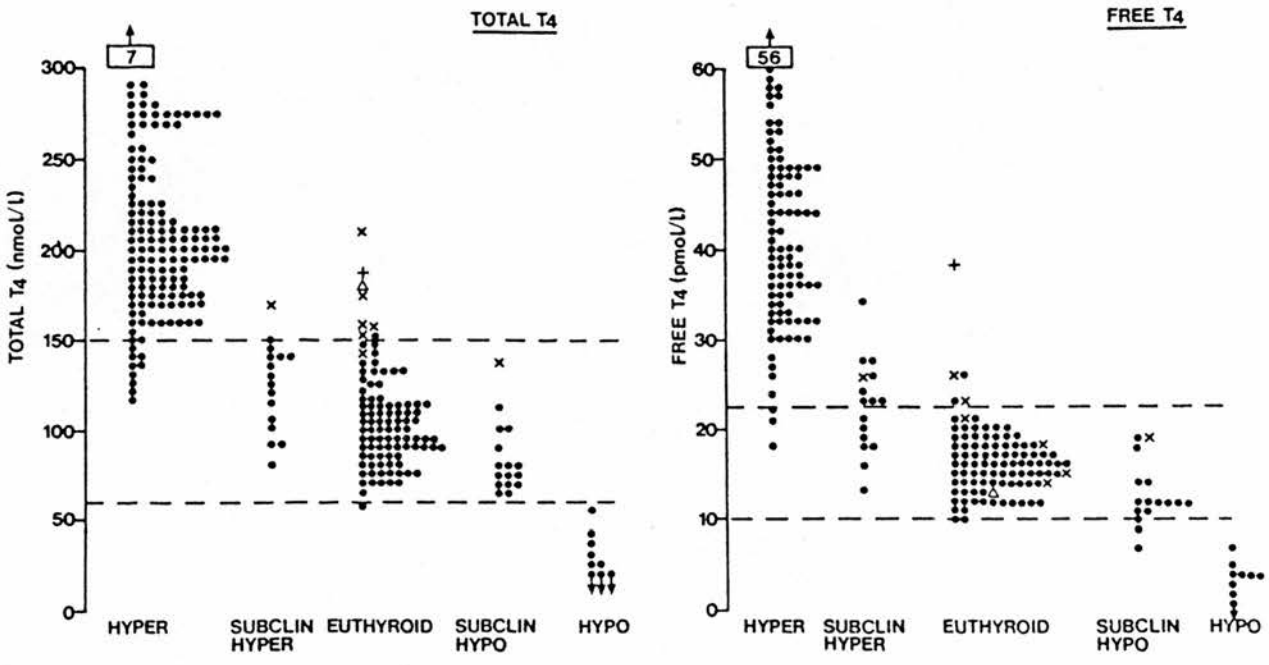


Figure 4.21 Total and Free T_4 Concentrations in Serum from 285 Patients from a Thyroid Clinic.

Results for women taking the OCP (x), a pregnant patient (Δ), and a patient with abnormal binding to albumin (+) are indicated.

Reference ranges (- - -).

fT_4 and not total T_4 there would have been a reclassification to overt hyperthyroidism in 9 of the 16 patients.

Most euthyroid patients with raised total T_4 due to altered protein binding (5/7) had normal fT_4 results.

As with total T_4, fT_4 was low in all 9 patients with overt hypothyroidism. Free T_4 was also low in 2 of the 16 patients considered to have subclinical hypothyroidism.

4.6.3 Free T_3 Compared with Total T_3 Concentrations

Free T_3 concentrations showed a greater rise than total T_3 in patients with hyperthyroidism being greater than 4 SD above the mean euthyroid value in 90% compared with 72% for total T_3 (Figure 4.22). The reference limits shown for total T_3 were those used to define patient categories whereas those for fT_3 (4.0-7.8 pmol/l) were derived from the 97 euthyroid patient results in this series. All but 4 of the 147 patients with overt hyperthyroidism had raised fT_3 levels but total T_3 concentrations were normal in 15, usually associated with drug treatment (e.g. propranolol) or non-thyroidal illness.

All euthyroid patients with raised total T_3 due to altered protein binding had normal fT_3 results.

In the 9 patients with overt hypothyroidism the fT_3 concentration was normal in two and total T_3 was normal in five. Free T_3 concentrations were normal in all patients with subclinical hypothyroidism.

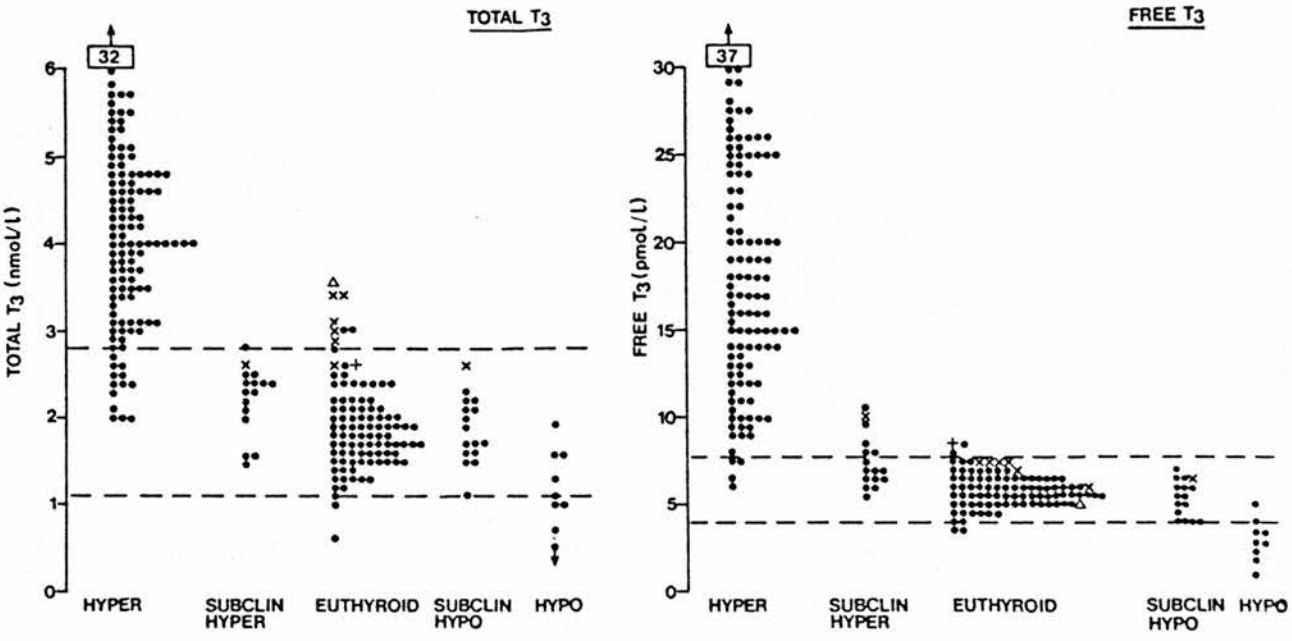


Figure 4.22 Total and Free T_3 Concentrations in Serum from 285 Patients from a Thyroid Clinic.

Results for women taking the OCP (x), a pregnant patient (Δ) and one with abnormal binding to albumin (+) are indicated.

Reference ranges (---).

4.6.4 The Predictive Value of the Thyroid Function Tests

Using the TRH test result only for patient categorisation (i.e. combining the overt and subclinical forms of disease as a single entity), the predictive values (PV) for TSH IRMA and thyroid hormone results were calculated (Table 4.12).

Table 4.12 The Predictive Values of Thyroid Function Tests in Patients from a Thyroid Clinic

Test	PV of Test Results for:-		
	Hyperthyroidism (overt & subclinical)	Euthyroidism	Hypothyroidism (overt & subclinical)
TSH IRMA	Undetectable PV = 1.0	0.14- 5.9 mU/l PV = 0.99	>5.9 mU/l PV = 1.0
fT_4	>22.5 pmol/l PV = 0.97	10-22.5 pmol/l PV = 0.79	<10 pmol/l PV = 0.92
fT_3	>7.8 pmol/l PV = 0.98	4.0-7.8 pmol/l PV=0.73	<4.0 pmol/l PV = 0.80
Total T_4	>150 nmol/l PV = 0.94	60-150 nmol/l PV = 0.68	<60 nmol/l PV 0.90
Total T_3	>2.8 nmol/l PV = 0.94	1.1-2.8 nmol/l PV = 0.63	<1.1 nmol/l PV = 0.67

The PV of an abnormal result was calculated as the probability that the result indicates the presence of disease i.e.:-

$$PV = \frac{\text{No. of abnormal tests in disease}}{\text{Total No. of abnormal results}}$$

Conversely, the PV of a normal result excluding the presence of disease was calculated as:-

$$\frac{\text{No. of normal results in the euthyroid patients}}{\text{Total No. of normal results}}$$

Cut-off points for calculating PVs are chosen to minimise overlap between patient categories and are usually estimated from a separate non-diseased population prior to the evaluation of the test (Galen & Gambino, 1975). The reference limits for total T_4 and T_3 by the routine diagnostic clinical chemistry service (Section 2.2.1) were therefore used for this purpose. Since there was minimal overlap between euthyroid and diseased groups for basal TSH IRMA, the absolute range found in the 97 euthyroid patients was used to define the cut-off points for this assay. There were no locally-derived reference values available for fT_4 and fT_3 in a separate population and the manufacturer's suggested reference ranges (fT_4 8.8-23.0; fT_3 2.9-8.9 pmol/l) did not minimise the overlap between diagnostic groups. The reference limits (mean±2SD) shown for the euthyroid group (Figures 4.21, 4.22), were therefore used to calculate PVs for the free hormone assays.

A result for TSH had the highest PV for both the presence and absence of all degrees of thyroid dysfunction. This test was significantly better than measurement of thyroid hormones for excluding thyroid disease thereby making it the most suitable first-line test in these patients. A normal, detectable TSH concentration would have the same significance as a normal TSH response to TRH in the exclusion of hyperthyroidism and primary hypothyroidism.

High results for fT_3 and fT_4 had similar PVs and both were raised by a similar proportion in hyperthyroid patients. However, as seen with total thyroid hormone measurements, a low fT_4 result had a higher PV for hypothyroidism than fT_3. Measurements of free thyroid hormones had higher PVs than total thyroid hormone measurements.

The PV of an individual result indicating the presence or absence of disease is dependent on the prevalence of disease in the population studied (Vecchio, 1966; Galen & Gambino, 1975). The calculated PVs shown in Table 4.12 therefore apply only to thyroid clinics referred a similar patient population to this study. In clinics with a lower prevalence of thyroid disease, the PV of a positive result would be lower. In addition, the sensitivity and specificity of each test shown in Table 4.13 might also change in a different patient population e.g. in hospital in-patients where changes in binding proteins, drug therapy etc., may affect the number of false positive and false negative results obtained. Thus, TSH IRMA may not necessarily provide the best first-line test in different clinical settings.

Table 4.13 The Sensitivity and Specificity of Thyroid Function Tests in Patients from a Thyroid Clinic

Test	Subclinical & Overt Hyperthyroidism	Euthyroidism	Subclinical & Overt Hypothyroidism
	Sensitivity	Specificity	Sensitivity
TSH IRMA	99%	100%	100%
FT_4	94%	94%	44%
FT_3	89%	95%	32%
Total T_4	84%	91%	36%
Total T_3	81%	90%	16%

$$\text{Sensitivity} = \frac{TP}{TP+FN} \times 100$$

$$\text{Specificity} = \frac{TN}{TN+FP} \times 100$$

TP = true positives
FN = false negatives
TN = true negatives
FP = false positives

4.7 SUMMARY AND DISCUSSION

In assessing the laboratory performance of the one-step analogue assays for free thyroid hormones, a range of imprecision and analytical inaccuracy was demonstrated (this suggests that a greater heterogeneity of performance would be expected with methods based on alternative principles). However, in a population with a high prevalence of thyroid disease, results by all of the analogue fT_4 kits correlated well with equilibrium dialysis and had similar diagnostic accuracy in samples from patients.

All of the fT_3 kits except Coat A Count produced similar patient categorisation. The measurement of free rather than total thyroid hormones had two major advantages: (a) results were less affected by alterations in TBG, (b) results had a higher PV for the presence and absence of thyroid disease. However, there was little evidence to support a major role for fT_3 measurement in addition to fT_4 in this patient group. These conclusions are supported by other studies of similar patients (Symons et al., 1983; Franklyn et al., 1984) and two recent reviews (Wilke 1986; Pearce & Byfield, 1986).

The coupling of analogue fT_4 measurement with the more sensitive measure of hypothyroidism, TSH (Irvine et al., 1973), could provide an attractive thyroid function 'test' for both clinician and laboratory. Indeed the use of the SimulTRAC fT_4/TSH assay could produce a large reduction (approximately 2/3) in the number of analyses performed in this context. In the evaluation of this assay, there were no patients with T_3-thyrotoxicosis but since the majority of such patients tend also to have increased fT_4, it is unlikely that they would be misclassified. The use of this dual RIA, could restrict the number of TRH tests performed in the thyroid clinic to the minority of patients who have normal fT_4 but low TSH results, in order to distinguish euthyroid patients from those with subclinical hyperthyroidism.

The two IRMA methods evaluated for TSH had acceptable precision with advantages to the laboratory arising from the wider working range compared to RIA. The Amerwell IRMA was particularly suitable for handling large workloads, a necessary pre-requisite for a first-line test. Lower TSH results by IRMA compared to RIA would be expected in view of the higher specificity of sandwich assays. However, the reason for the discrepancy between Amerwell and Boots-Celltech TSH values is less clear. Measurement of TSH by immunofluorimetric assay has also yielded lower results (28%) than the Boots-Celltech IRMA (Paterson et al., 1985) leading to the suggestion that different monoclonal antibodies may have different relative specificities for endogenous and standard TSH. The lower Amerwell reference range is also more in keeping with those quoted for other new immunometric assays (Weeks et al., 1984; Kaihola et al., 1985; Clark & Price, 1986) and research methods (Durham, 1985).

The availability of TSH immunometric assays with increased sensitivity and specificity compared to RIA has raised the possibility that basal TSH could be used as a first-line test of thyroid status making the TRH test redundant. The absence of a TSH response to TRH is the most sensitive indicator of thyroid hormone excess (Snyder & Utiger, 1972a) and, therefore, the demonstration of the

resolution of hyperthyroid and euthyroid basal concentrations of TSH IRMA and the strong relationship between the basal TSH IRMA and the TSH response to TRH in euthyroid patients (Figure 4.23A), even for basal values undetectable by RIA (Figure 4.23B), provides evidence that basal TSH IRMA gives a reliable indicator of thyrotroph activity throughout the entire spectrum of thyroid disease.

Although an absent TSH response to TRH is consistent with hyperthyroidism, this may also be found in other clinical situations as discussed earlier (Section 1.3.4). It is likely, therefore, that basal TSH (IRMA) might be abnormal in other circumstances. The performance of basal TSH by IRMA and free thyroid hormone tests by analogue RIA in other clinical situations, and the development of a new thyroid testing strategy applicable to the thyroid clinic (for diagnosis and monitoring therapy) and for screening pregnant women, hospital inpatients and geriatric patients are the subjects of subsequent chapters.

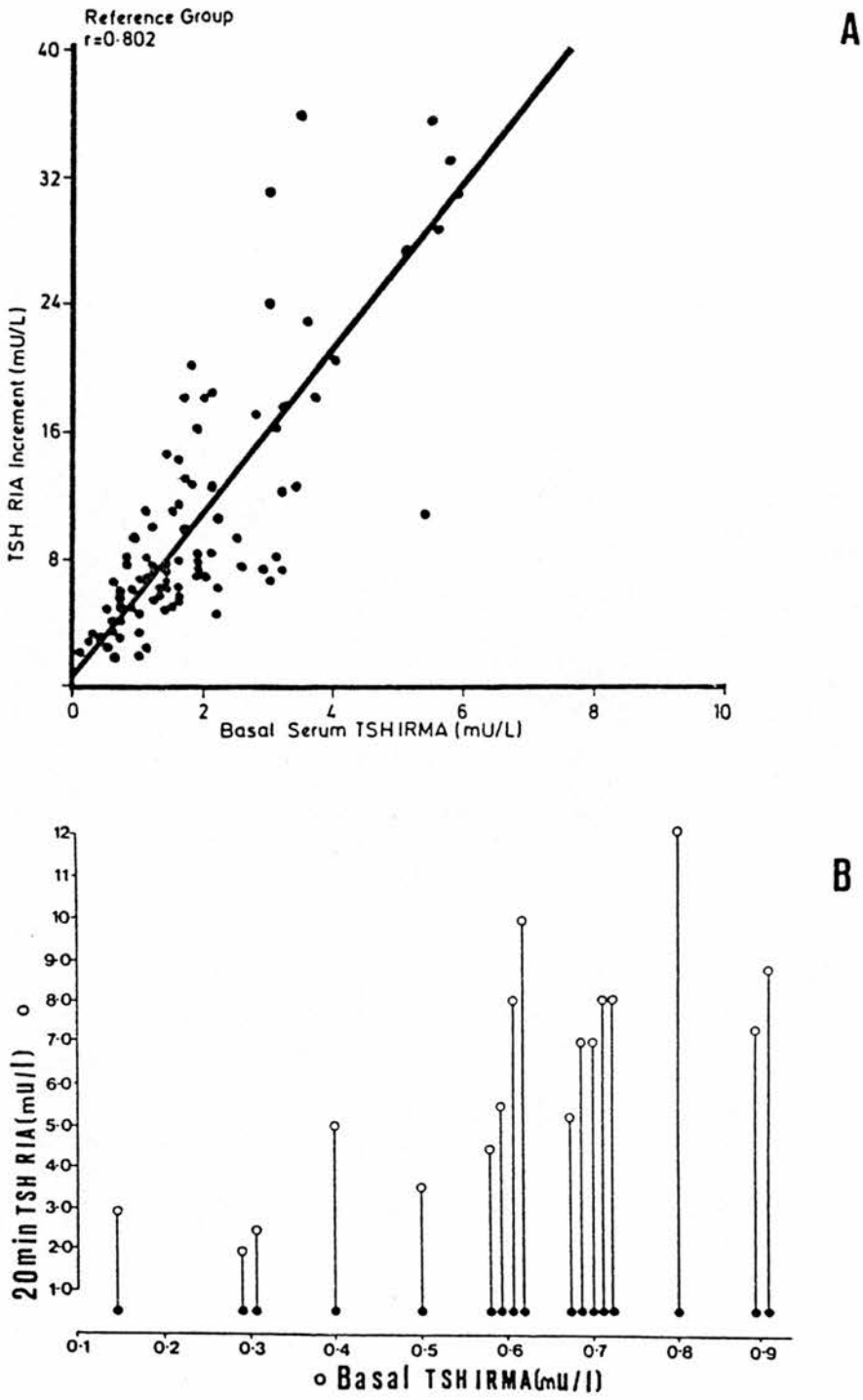


Figure 4.23 The Relationship Between TSH Responsiveness to TRH and Basal TSH Measured by Boots-Celltech IRMA.

(A) The euthyroid reference group
(B) Euthyroid patients with basal TSH undetectable by RIA.

Chapter 5

THYROID FUNCTION TESTS IN PREGNANCY AND IN OTHER SITUATIONS OF ALTERED PROTEIN-BINDING OF THYROID HORMONES

5.1 THYROID FUNCTION TESTS IN PREGNANCY

The diagnosis of hyperthyroidism in a pregnant woman based purely on clinical examination may be difficult because increased heart rate, heat intolerance and tiredness are common features of both hyperthyroidism and pregnancy. Biochemical tests of thyroid status may be of considerable value for effective diagnosis particularly since appropriate therapy is important for the health of the mother and the development of the foetus (Woods et al., 1984). An understanding of the normal function and control mechanisms operating on the thyroid during pregnancy is necessary for the correct interpretation of biochemical tests.

Measurement of free rather than total thyroid hormone concentrations should accurately reflect thyroid status in pregnancy. However, it is known that FT_4I values remain unchanged fortuitously, since the T_3-uptake methods fail as TBG rises to high levels (Whitworth et al., 1982). Early studies using indirect dialysis methods have reported unchanged, increased and decreased values (Ekins, 1979); direct dialysis has yielded unchanged (Symons, 1983) and decreased values (Yeo et al., 1977b; Lewis,1979; Weeke et al., 1982; Giles, 1982; Helenius & Liewendahl, 1983) and measurements by analogue RIA have shown decreased concentrations as pregnancy progresses (Whitworth et al., 1982; Tuttlebee, 1982;

Symons et al., 1983; Hopton et al., 1983; Wilke, 1983; Franklyn et al., 1983). Measurements using microencapsulated antibodies, by contrast, show little or no change with gestation (Boss & Kingstone, 1981; Amino et al., 1983). Since recent studies have shown that measurement of free thyroid hormones may be spuriously affected by changes in serum albumin and NEFA levels (Bayer 1983a & b; Amino et al., 1982 & 1983; Stockigt et al., 1983) both of which are known to change with gestation (Amino et al., 1983; Burt, 1960), the true free thyroid hormone concentrations circulating in late pregnancy are still debated.

In pregnancy, the TRH test is contra-indicated since it may produce undesirable reactions in a small number of patients (Editorial, 1984). In addition, some RIA methods for TSH show significant cross-reactivity of the antisera to high circulating levels of HCG. The measurement of TSH by sensitive IRMA may circumvent the problems of assay specficity and allow the differentiation of hyperthyroidism from euthyroidism in pregnancy without TRH injection.

5.1.1 Subjects Studied

Ninety-three women in the first (7-12 wk, n=37), second (13-27 wk, n=32) and third (28-40 wk, n=24) trimesters of pregnancy were studied. Serum samples were taken at their afternoon visit to the antenatal clinic.

5.1.2 Concentrations of Total Thyroid Hormones, TBG and Albumin in Serum

Total T_4, total T_3 and TBG concentrations increased by 8%, 15% and 41%, respectively as pregnancy progressed (Figure 5.1 and Table 5.1) whereas the T_4/TBG ratio decreased. Albumin concentrations decreased by 16% (i.e. an average of 7 g/l) between the first and third trimesters.

5.1.3 Free Thyroid Hormone Concentrations by Analogue RIA and Equilibrium Dialysis

There was a significant decrease in the concentration of both fT_4 and fT_3 during pregnancy although there was a marked difference in the magnitude of this fall when measured by analogue RIA as compared to dialysis (Figures 5.2 and 5.3). In addition, analogue RIA methods produced by different manufacturers showed differing degrees of change as pregnancy progressed (Table 5.1). This was particularly evident for fT_3 where low results were found in 1% and 35% of women using the Coat A Count and Becton Dickinson kits, respectively. Although no women had fT_4 or fT_3 values below reference limits by dialysis-RIA, several low values were found by analogue-RIA depending on the kit (Table 5.1).

The correlations obtained between fT_4 levels measured by dialysis and analogue-RIA in the 93 pregnant women were, in general, less significant than those in 62 non-pregnant euthyroid outpatients (Table 5.2), in view of

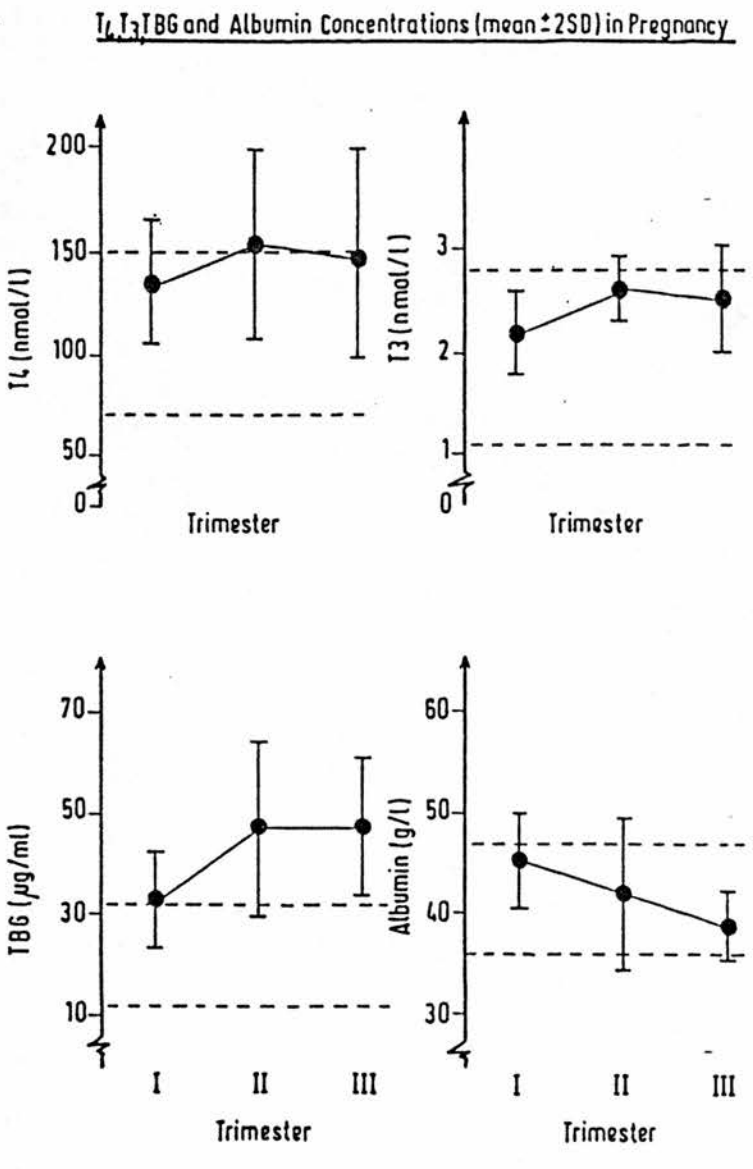


Figure 5.1 Total T_4, T_3, TBG and Albumin Concentrations (Mean ± 2SD) in Pregnancy.

Table 5.1 Thyroid Function Tests, Serum Albumin and NEFA Concentrations in the Three Trimesters of Pregnancy

Test		Trimester I	Trimester II	Trimester III	Mean % Change I-III	% Subnormal Results
	Total T_4 (nmol/l)	136(19.5)	152(22.8)[b]	147(24.9)[c]	+8	-
	Total T_3 (nmol/l)	2.2(0.4)	2.6(0.3)[a]	2.5(0.5)[b]	+15	-
	TBG (mg/l)	33.0(4.8)	46.8(7.9)[a]	46.5(7.3)[a]	+41	-
	T_4/TBG (nmol/mg)	4.1(0.65)	3.3(0.64)[a]	3.3(0.62)[a]	-19	-
Free T_4 (pmol/l)	Dialysis	13.4(1.8)	12.1(1.9)[b]	11.3(1.6)[a]	-16	0
	Amerlex	16.8(1.6)	14.7(3.1)[a]	12.6(2.2)[a]	-25	5
	Amerlex-M	16.1(1.6)	14.3(2.9)[a]	11.8(2.1)[a]	-27	4
	Corning Magic	23.2(2.0)	20.2(3.9)[a]	17.6(2.0)[a]	-24	12
	Coat A Count	16.5(2.3)	14.1(3.4)[a]	12.1(2.1)[a]	-27	14
	Becton Dickinson	12.8(1.8)	11.5(2.5)[c]	9.7(1.6)[a]	-24	4
Free T_3 (pmol/l)	Dialysis	5.6(0.8)	5.1(0.9)[c]	5.1(0.9)[c]	-9	0
	Amerlex	6.2(0.8)	5.0(1.0)[a]	4.0(0.5)[a]	-35	14
	Amerlex-M	5.6(0.8)	4.7(0.8)[a]	3.8(0.7)[a]	-35	14
	Coat A Count	5.0(1.0)	4.8(0.9)	4.3(0.8)[b]	-14	1
	Becton Dickinson	6.8(1.2)	5.8(1.0)[a]	4.8(0.9)[a]	-29	35
	Albumin (g/l)	45 (2.4)	42 (3.3)[a]	38 (1.7)[a]	-16	-
	NEFA (mmol/l)	0.44(0.26)	0.34(0.17)	0.36(0.11)	NS	-
TSH (mU/l)	In-house RIA	2.8(0.7)	2.5(0.6)[c]	2.6(0.6)	NS	-
	Becton Dickinson RIA	3.2(0.9)	3.1(1.1)	3.3(1.0)	NS	-
	Boots-Celltech IRMA	1.0(0.8)	1.3(1.0)	1.6(0.9)[c]	+65	3

Mean values (±SD) and the mean % change between trimesters I and III are shown for each test with the % of low results for thyroid function tests. Significant differences (t-test) from first trimester levels are indicated by: a) $p<0.001$, b) $p<0.01$ and c) $p<0.05$. NS = not significant.

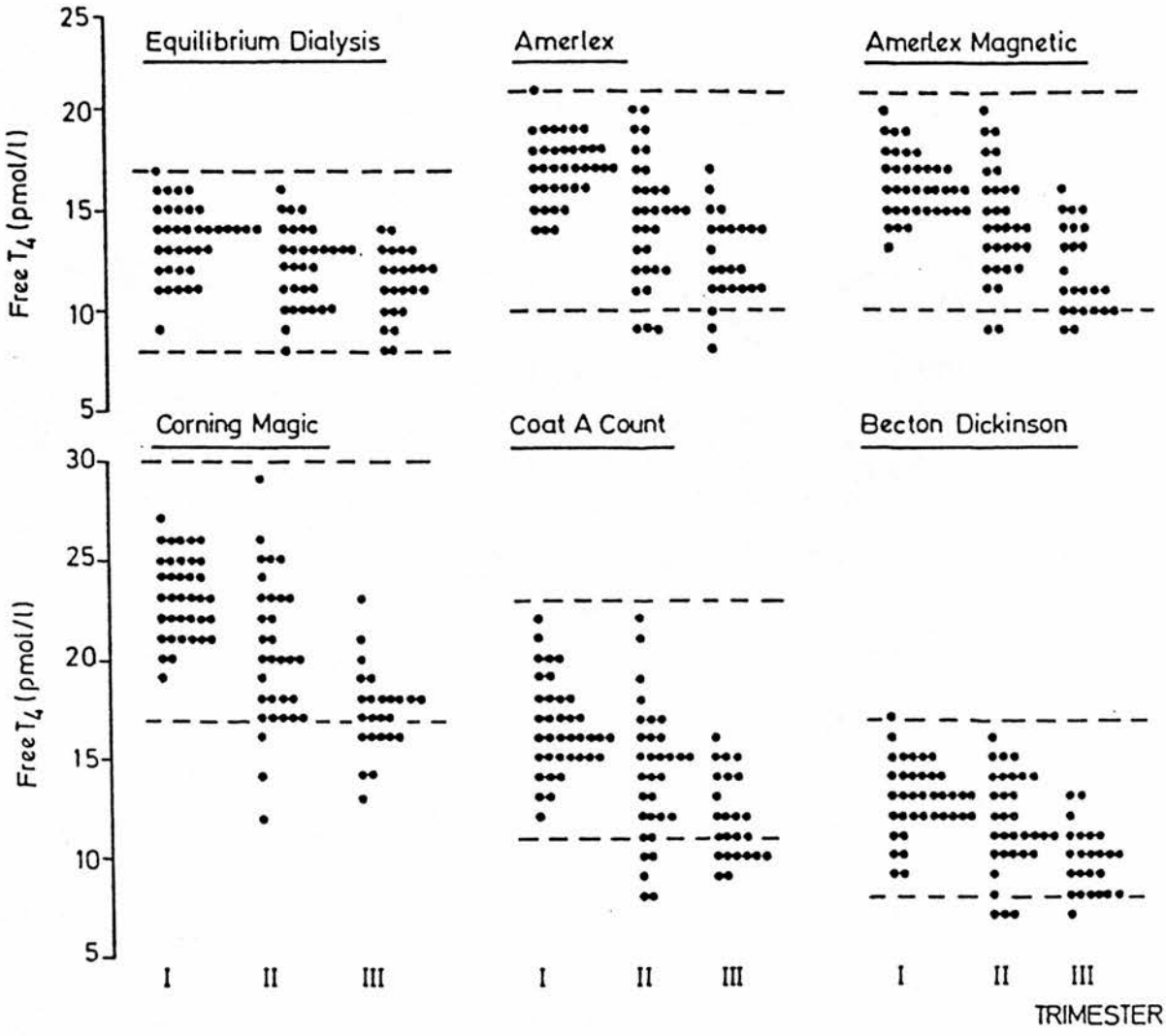


Figure 5.2 Free T4 Concentrations in the Three Trimesters of Pregnancy.

Reference ranges are indicated by dashed lines.

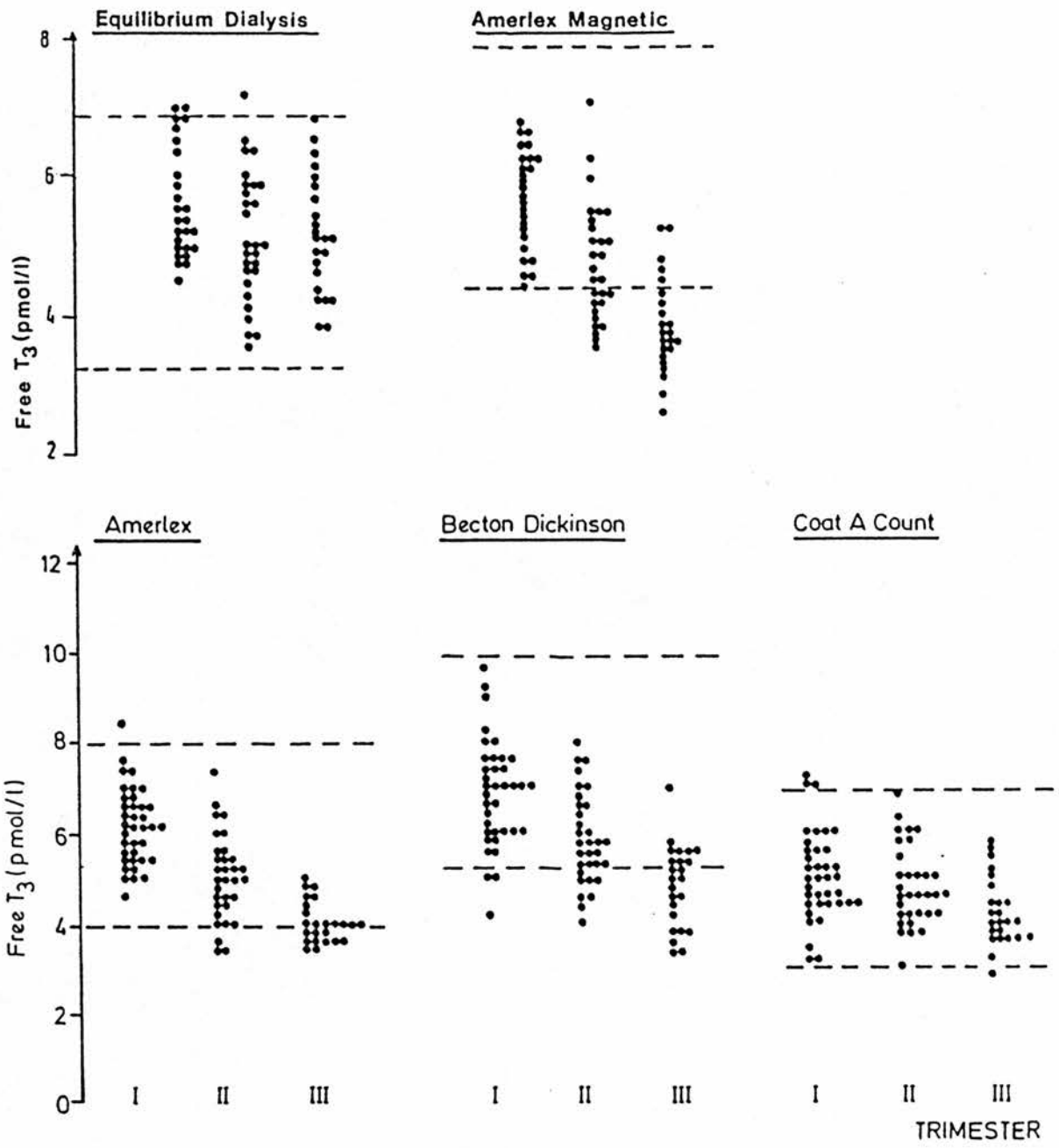


Figure 5.3 Free T_3 Concentrations in the Three Trimesters of Pregnancy.

Reference ranges are indicated by dashed lines.

the smaller group in the latter. This was not the case with fT_3 measurements where there was poorer correlation between the analogue methods and dialysis in both groups over the narrow concentration range studied (Table 5.2). The Becton Dickinson fT_3 kit gave the poorest correlation with dialysis values in pregnancy.

Table 5.2 Correlations Between Free Thyroid Hormome Measurements by Dialysis and Analogue RIA in Pregnant and Non-pregnant Outpatients

Analogue RIA	Correlation with Dialysis Values (r)	
	Pregnant Women (n=93)	Euthyroid Patients (n=62)
Free T_4:		
Amerlex	0.620[a]	0.621[a]
Amerlex-M	0.598[a]	0.649[a]
Corning Magic	0.572[a]	0.665[a]
Becton Dickinson	0.580[a]	0.594[a]
Coat A Count	0.599[a]	0.518[a]
Free T_3:		
Amerlex	0.391[a]	0.279
Amerlex-M	0.328[b]	0.291
Becton Dickinson	0.257	0.161
Coat A Count	0.470[a]	0.155

a) $p<0.001$, b) $p<0.01$

5.1.4 Correlations Between Free Thyroid Hormone Concentrations and Serum Albumin and NEFA Levels

Concentrations of albumin in serum decreased by 16% during pregnancy but NEFA levels did not change significantly (Table 5.1). In contrast to serum albumin, none of the free thyroid hormone measurements correlated with NEFA levels in these non-fasting subjects. However,

comparison of fT_4 or fT_3 with any other serum constituent which also falls during pregnancy is likely to produce a good correlation. Of more interest was the strong positive correlation ($p<0.001$) observed between albumin and free thyroid hormones in the shorter time-period of the second trimester with the analogue kits, but not with equilibrium dialysis (Table 5.3). Highly significant correlations ($p<0.001$) with albumin were also observed in non-pregnant euthyroid out-patients for fT_3 measured by the Amerlex-M and Becton Dickinson kits. There was no correlation between fT_4 and albumin in these patients. However, the range of albumin concentrations in this group (43-53 g/l) was less than in the second trimester of pregnancy (36-50 g/l).

Table 5.3 Correlation Between Free Thyroid Hormone and Serum Albumin Concentrations in Pregnant and Non-pregnant Subjects

Method	Correlation with albumin (r)			
	Trimester of Pregnancy			Non-pregnant Euthyroid Out-patients
	I	II	III	
n	37	32	24	63
Free T_4				
Dialysis-RIA	-0.045	0.320	0.147	-0.146
Amerlex	0.018	0.575[a]	0.110	0.144
Amerlex-M	0.267	0.613[a]	-0.040	-0.010
Corning Magic	0.192	0.608[a]	-0.023	0.053
Becton Dickinson	0.156	0.572[a]	-0.196	0.126
Coat A Count	0.305	0.723[a]	0.111	0.068
Free T_3				
Dialysis-RIA	-0.199	0.178	-0.504	0.324
Amerlex	0.405	0.663[a]	0.325	0.295
Amerlex-M	0.270	0.649[a]	0.365	0.470[a]
Becton Dickinson	0.587[a]	0.481[b]	0.235	0.506[a]
Coat A Count	0.249	0.344	0.284	0.181

Significant correlations a) $p<0.001$, b) $p<0.01$

5.1.5 The In vitro Effect of Added Albumin on Free Thyroid Hormone Values in Serum

The effect of albumin on free thyroid hormone levels measured by dialysis-RIA and by the Amerlex kits was examined by adding 3 - 19 g/l human serum albumin (Behring, Marburg, West Germany of 100% electrophoretic purity) in 0.15 mM NaCl to aliquots of a low-albumin pool of serum collected from hospital inpatients, to give a final albumin concentration range of 30 - 50 g/l. Insufficient reagents were available to repeat the experiment with the other free hormone kits.

Free thyroid hormone levels did not change significantly when measured by dialysis-RIA whereas linear, dose-dependent increases in fT_4 and fT_3 were observed using the Amerlex analogue methods (Figure 5.4). The mean decrease in albumin of 7 g/l which occurred during pregnancy could, therefore, account for a decrease of 1.8 pmol/l (11%) in fT_4 concentration and 0.75 pmol/l (13%) in fT_3 concentration by Amerlex assay, as pregnancy progressed. Despite the inclusion of an "albumin blocker" in the Amerlex-M fT_4 kit by the manufacturer, results were affected by added albumin in a similar way to the Amerlex kit. When the changes in free hormone levels in pregnant women (Table 5.1) were corrected for this albumin effect, the percentage changes observed in fT_4 during pregnancy when measured by analogue RIA were in good agreement with

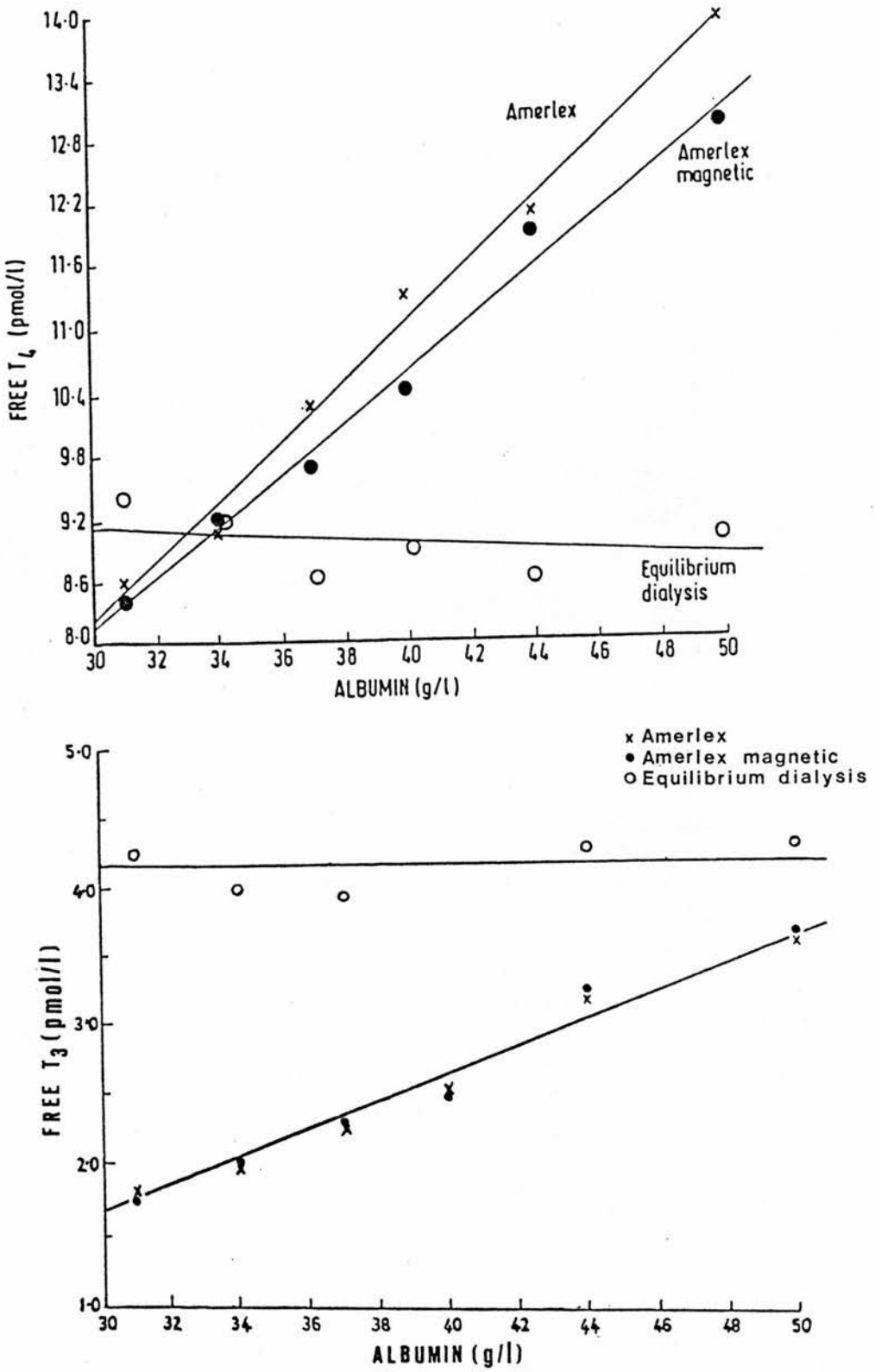


Figure 5.4 The Effect of Added Human Serum Albumin on Free Thyroid Hormone Concentrations in a Human Serum Pool Using Dialysis and Analogue RIA Methods.

those measured by dialysis-RIA. However, there was still marked disparity in fT_3 levels between the analogue and dialysis methods (Table 5.4).

Table 5.4 The Change in Free Thyroid Hormone Levels by Analogue RIA after Correction for the Calculated Albumin Effect

	Mean Change Between Trimesters I - III	
	fT_4	fT_3
Analogue RIA	-27%	-35%
Change due to Albumin	-11%	-13%
Analogue RIA-corrected	-16%	-22%
Dialysis-RIA	-16%	- 9%

5.1.6 Basal TSH Measurements in Pregnancy

Results for SimulTRAC free T_4/TSH RIA measurements in pregnancy are shown in Figure 5.5(A), demonstrating normal TSH concentrations in the presence of low fT_4 results by analogue RIA in the third trimester. With this dual assay, therefore, interpretative problems with respect to low fT_4 values in pregnancy would be reduced. However, SimulTRAC TSH values in these pregnant women were signficantly higher ($p<0.001$; mean 4.0 mU/l, range 1.7 - 6.3) than those found in non-pregnant euthyroid outpatients (Figure 4.12) suggesting some residual cross-reactivity problems due to high HCG levels in pregnancy. This problem, coupled with the marked lowering of fT_4 values by analogue assay in pregnancy, could potentially produce normal SimulTRAC results in the presence of mild hyperthyroidism.

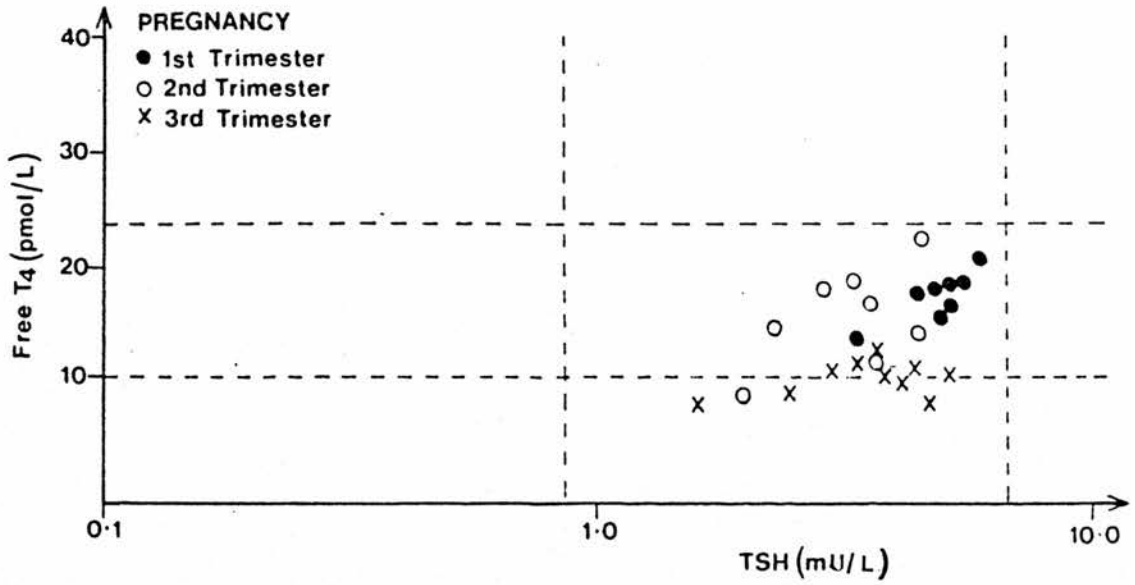


Figure 5.5(A) SimulTRAC Free T4/TSH RIA Results in Pregnancy.

Reference ranges are indicated by dashed lines.

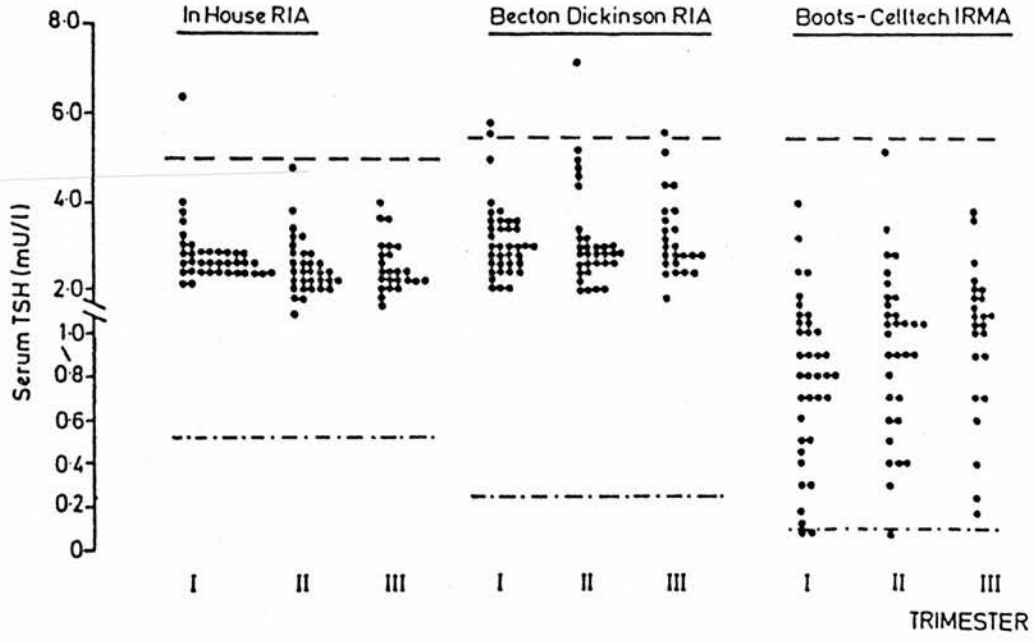


Figure 5.5(B) Basal TSH Concentrations by RIA and IRMA in Pregnancy.

The upper reference limit (---) and the mean minimum detection limit for each assay (—·—) are as indicated.

Results for basal TSH by single-analyte RIA and the Boots-Celltech IRMA are shown in Figure 5.5(B). The TSH IRMA results were lower than results by RIA in all trimesters, and 3 values fell below the mean detection limit, in early pregnancy (Table 5.5). There was a significant increase in mean TSH IRMA values between the first and third trimesters, the Becton Dickinson kit showed no change, and values by the in-house assay significantly decreased between the first and second trimesters (Table 5.1). The fact that no TSH results were undetectable by RIA suggests a specificity problem with these assays. Measurement of TSH in sera from 20 women in the first trimester using the Amerwell IRMA produced similar results to non-pregnant euthyroid values but three women had low-detectable values at less than 13 weeks gestation (Table 5.5).

5.1.7 Concentrations of HCG and TSH in Pregnancy

Measurements of HCG by RIA (Vaitukaitis et al., 1972) were performed by staff of the Centre for Reproductive Biology, Edinburgh. This method employed an antiserum directed against the β subunit of HCG, the 2nd HCG IRP for standardisation and a double-antibody separation system.

Concentrations of HCG peaked in the first trimester of pregnancy (Figure 5.6(A)) with a subsidiary rise in the third trimester. A low-order inverse correlation

Table 5.5 Subjects with Low TSH IRMA Values in Pregnancy

Subject	Gestation (wks)	TSH IRMA (mU/l)		Dialysis-RIA (pmol/l)		HCG
		Boots-Celltech (0.14-5.9)	Amerwell (0.36-4.5)	fT_4 (8 - 17)	fT_3 (3.2 - 6.7)	(U/l x 10^3)
1	11	<0.10	0.40	12.4	6.4	30.0
2	12	<0.10	0.19	14.7	6.4	39.6
3	9	0.10	0.19	15.3	6.5	54.8
4	11	0.12	0.28	10.9	5.9	32.2

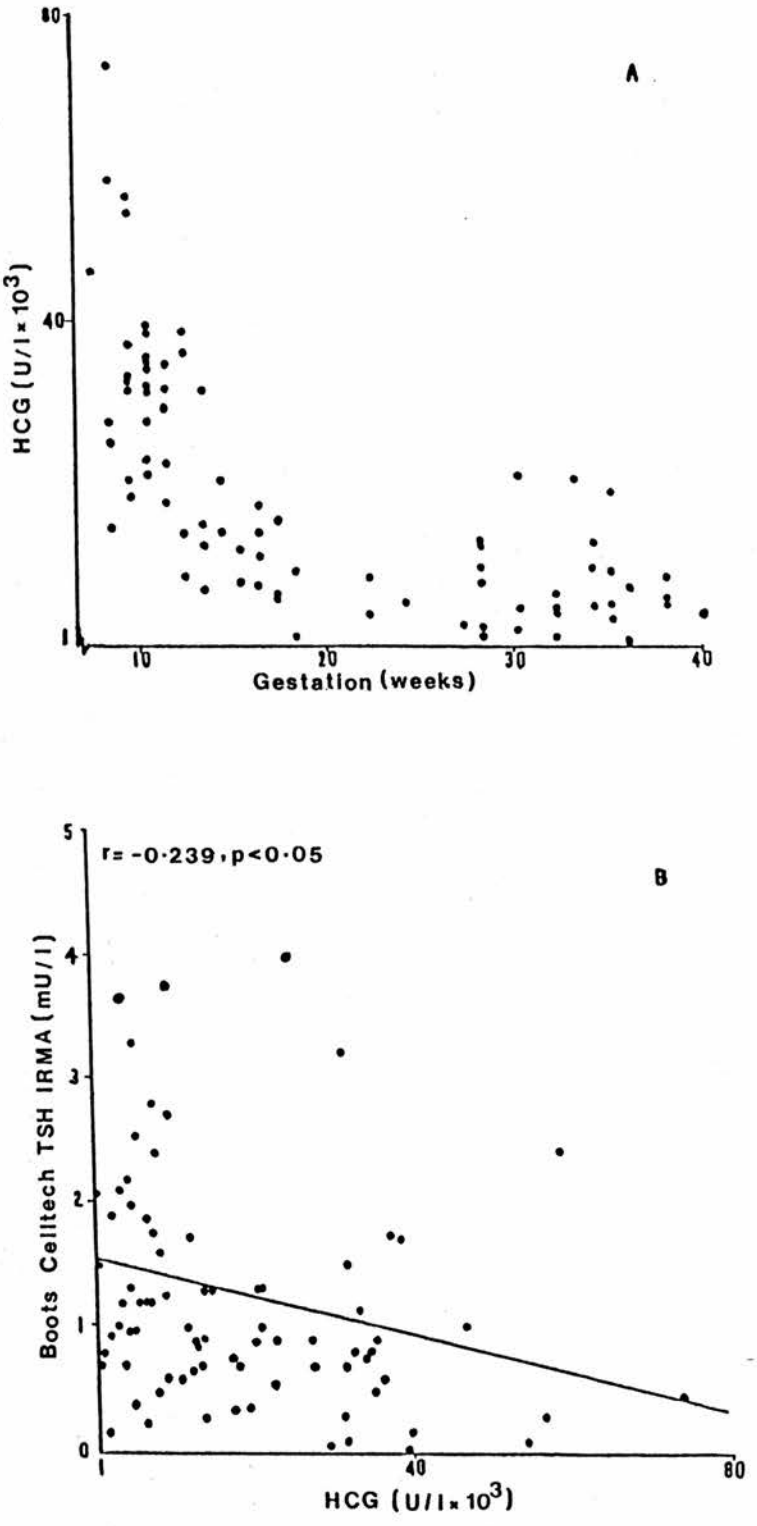


Figure 5.6 The Concentrations of HCG during Pregancy (A) and the Relationship with TSH Concentrations Measured by Boots-Celltech IRMA (B).

($p<0.05$) was observed between Boots-Celltech TSH IRMA values and HCG in pregnancy, Figure 5.6B. However, patients with undetectable TSH IRMA (Table 5.5) did not have the highest HCG concentrations.

5.1.8 Summary and Discussion

Abnormal results for thyroid function tests were found frequently in normal pregnancy. Measurements of free rather than total thyroid hormones were of greater value but often produced low results in the hypothyroid range by analogue assay. The decrease in free thyroid hormone concentrations as pregnancy progressed was exaggerated by analogue RIA compared to equilibrium dialysis, in agreement with the study of Helenius & Liewendahl (1983) but in contrast to that of Jackson & Ekins (1986), who studied fewer patients. This decrease was also greater than that of the T_4:TBG ratio, unlike a previous report (Whitworth *et al.*, 1982) and, therefore, did not support the accuracy of the analogue methods in pregnancy.

The difference between fT_4 values in pregnancy by the alternative methodologies could not be attributed to raised NEFA concentrations in late pregnancy in this study. The increase in NEFA reported by Burt (1960) was 0.4 mmol/l above the mean of non-pregnant controls. It is unlikely that such a small rise would be discernible in the present study as non-fasting samples were taken. Wilkins & Midgley (1982) also argued against high NEFA

levels in pregnancy causing spuriously low values by analogue RIA and the recent work of Mendel et al. (1986) has shown that increases in NEFA greater than 2 mmol/l are necessary for fT_4 results by equilibrium dialysis to be significantly affected.

A strong correlation between albumin and fT_4 concentrations was observed in the second trimester using the analogue kits, but not with equilibrium dialysis. This may reflect the poorer precision of the dialysis method and cannot be interpreted as an albumin effect on the kit assays (Wilkins & Midgley 1982; Midgley & Wilkins, 1983) although other workers have done so (Amino et al., 1983; Hata et al., 1987). However, the fact that this correlation was present for certain analogue assays in euthyroid non-pregnant out-patients supports the view of Wilke (1984) that some assays may be unduly influenced by physiological changes in the serum albumin concentration in pregnancy. The demonstration in vitro of increasing analogue fT_4 and fT_3 values with increments of serum albumin within the reference range (36-46 g/l), also supports this view. Correction of analogue values for the measured albumin effect produced closer agreement with the dialysis method, particularly for the change in fT_4 levels in pregnancy. This in vitro effect has been shown by others (Amino et al., 1983; Bayer, 1983a; Stockigt et al., 1983; Liewendahl et al., 1984) but

Wilkins et al. (1985) reported lower effects on the Amerlex kits than found here and also suggested that this effect was minimised by inclusion of the "albumin blocker". This was not confirmed in the present study for the Amerlex-M fT4 kit. The fact that results for clinical samples show less albumin-dependence has led Wilkins et al. (1985) to suggest that alterations in the binding properties of commercial preparations of albumin may be the source of this discrepancy, but there is no experimental evidence, as yet, to support this.

The fact that TSH concentrations were not elevated above normal in the third trimester confirmed the adequacy of free thyroid hormone levels as sensed by the pituitary gland. High HCG levels may cause cross-reaction problems in TSH RIA and interference in TSH IRMA. The former problem is well-recognised and often reduced by prior absorption of the anti-TSH antiserum with LH so that TSH RIA can be used effectively to detect hypothyroidism if it occurs in pregnancy, but cannot be used to determine physiological changes in TSH secretion as pregnancy progresses. Measurement of TSH by IRMA may be affected by high HCG levels due to disruption of the monoclonal antibody sandwich (Figure 5.7) giving rise to spuriously low levels. Kreutzer et al. (1986) confirmed this problem with the Boots-Celltech IRMA. In pregnancy, this interference is estimated by the manufacturer to produce a 13%

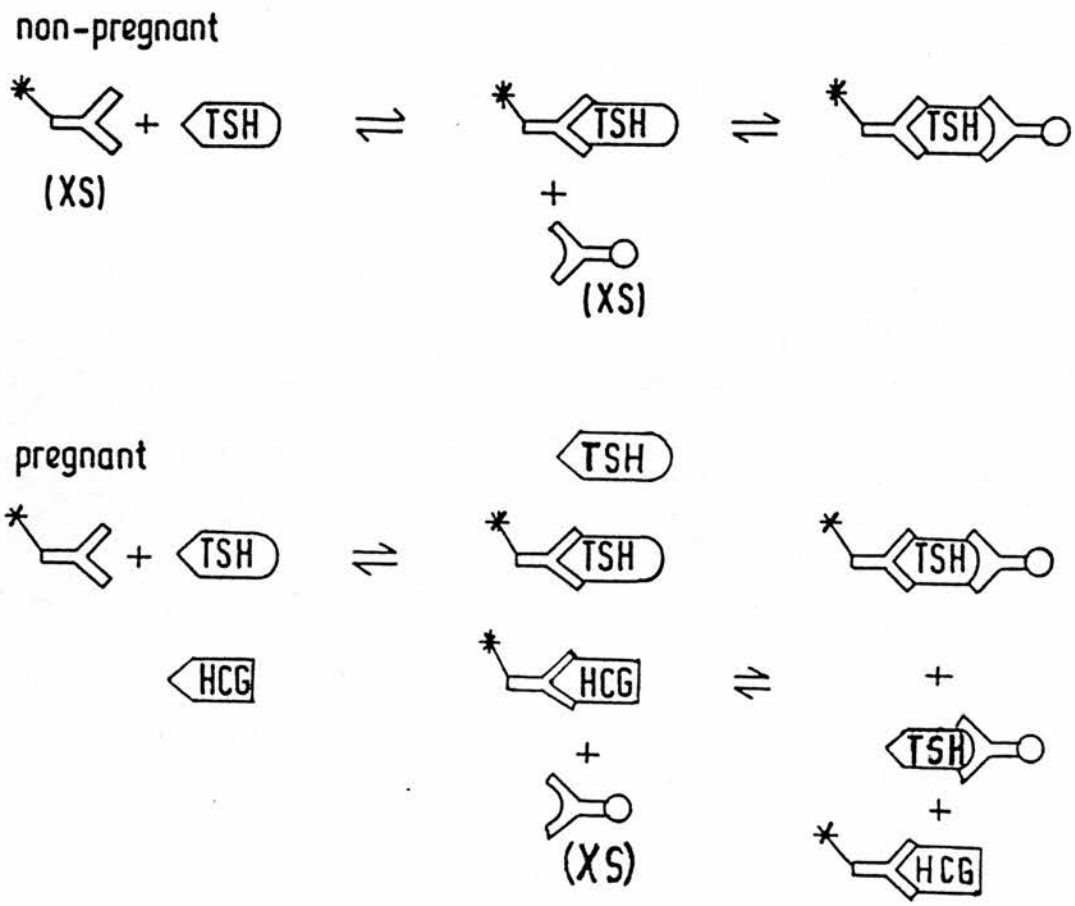


Figure 5.7 The Interference of HCG on TSH Levels Measured by Boots-Celltech IRMA.

HCG is recognised by the radiolabelled monoclonal antibody. At high HCG concentrations there is insufficient radiolabelled monoclonal to bind to TSH. Less radiolabel becomes bound to the solid-phase and this is interpreted as a low TSH value.

decrease in TSH values, whereas negligible interference is quoted for the Amerwell IRMA. The data shown here suggest a greater lowering of Boots-Celltech TSH values than accountable by methodological interference alone. However, the inverse correlation demonstrated between the Boots-Celltech TSH and HCG values in pregnancy could be due either to methodological interference in the TSH assay or a physiological decrease in TSH secretion when HCG levels were highest. Recently, Newman et al. (1985), using an in-house TSH IRMA, calculated that the negative interference by HCG was not sufficient to account for the low and undetectable values found in early pregnancy. Low TSH values were reported in early studies using sensitive RIA methods (Braunstein & Hershman, 1976; Yamamoto et al., 1979; Weeke et al., 1982) and, more recently, reduced responses to TRH in the first few weeks of pregnancy have also been demonstrated (Guillaume et al., 1985). An undetectable TSH (IRMA) level in early pregnancy may therefore be of limited importance in the diagnosis of hyperthyroidism.

It is known that HCG has some thyrotrophic activity (Nisula & Ketelslegers, 1974) and this is affected by the degree of its glycosylation (Amr et al., 1984). In the first trimester, HCG levels increase and the hormone is more highly glycosylated (Fein et al., 1980) which has led to the theory that HCG partially drives the maternal thyroid gland (Yamamoto et al., 1979; Weeke et al., 1982; Guillaume et al., 1985).

5.2 THYROID FUNCTION TESTS IN OTHER ALTERED PROTEIN-BINDING STATES

5.2.1 Alterations in TBG Concentration

In patients with raised TBG concentrations due to the OCP, free thyroid hormone concentrations by analogue RIA generally fall within reference limits as discussed in Chapter 4, and this has been confirmed in several larger studies (Waud et al., 1983; Kubasik et al., 1983; Witherspoon et al., 1985). Such measurements, therefore, offer a distinct advantage over total thyroid hormone measurements. However, as shown for the FT4I (Burr et al., 1977), there is some evidence that measurements by analogue RIA may not perform so well when there are gross changes in TBG (Waud et al., 1983; Jackson & Ekins, 1986).

Results for thyroid function tests and TBG in patients with raised total T4 not due to oestrogens are shown in Table 5.6 (patients 1-5). The high total T4 concentrations found on routine screening in patients 1 - 4 raised the possibility of hyperthyroidism, although these patients were clinically euthyroid. One patient (patient 5) was diagnosed previously as having autoimmune hypothyroidism on the basis of TSH measurements, autoantibody titres and free thyroid hormone concentrations and is currently being maintained on 100 μg T4 daily. All of these patients had raised TBG concentrations to a greater or lesser extent and the majority (4/5) were women.

Table 5.6 Thyroid Function Tests in Patients with Altered TBG Concentrations

Patient	Sex	Age (Yrs)	Clinical Impression	Total T_4 (nmol/l)	Total T_3 (nmol/l)	TBG (mg/l)	Amerlex fT_4 (pmol/l)	Amerlex fT_3 (pmol/l)	Boots-Celltech TSH IRMA (mU/l)
				Reference Range 60-145	1.1-2.7	*M 9-31 F10-32	Reference Range 10-22.5	4.0-7.8	Reference Range 0.14-5.9
1	F	47	Euthyroid	180	2.5	37	18	4.3	1.8
2	M	46	Euthyroid	159	2.4	31	14	5.4	1.9
3	F	55	Euthyroid	200	3.1	42	17	5.3	3.3
4	F	56	Euthyroid	199	2.3	34	22	7.6	1.2
5	F	62	Hypothyroid on T_4	286	4.0	83	20	6.2	0.8
6	M	24	Euthyroid	30	1.1	4	14	4.9	1.2
7	M	6	Euthyroid	45	1.5	8*	13	ND	1.2
8	F	32	Euthyroid	33	1.0	6	16	5.4	2.2
9	M	51	Euthyroid	<20	1.0	2	18	6.3	0.5
10	M	61	Hyperthyroid	120	2.4	10	42	7.5	<0.07

*Kit Manufacturers quote higher reference range of 11-37 mg/l for TBG in childhood.
ND = Not determined.

Measurements of free thyroid hormones and TSH IRMA confirmed the euthyroid status of these patients.

Results for patients found to have low concentration of TBG (patients 6-10) are also detailed in Table 5.6. The majority (4/5) in this case were male, and euthyroidism was confirmed in patients 6-9 by the measurement of normal free thyroid hormone and basal TSH concentrations. One patient (patient 10) was suspected clinically to be hyperthyroid but had a normal total T_4 concentration. The raised fT_4 concentration by analogue-RIA was confirmed by equilibrium dialysis at 50 pmol/l and the patient had an undetectable TSH IRMA and absent TSH response to TRH. The finding of the TBG concentration at the lower limit of normal in this patient may explain the normal total T_4 concentration in the presence of clinical hyperthyroidism.

The good performance of the Amerlex assays in such patients confirms the findings of Wilke (1982), Ramsden et al. (1982), Byfield et al. (1983) and Franklyn et al. (1983). Jackson & Ekins (1986) however, found some divergence from dialysis values which varied between different analogue and non-analogue assays.

5.2.2 Abnormal Thyroid Hormone Binding to Albumin

During the course of this thesis, some patients were identified who were clinically euthyroid but who had

increased fT_4 values by analogue RIA with high or borderline-high total T_4 concentrations. Thyroid function test results for these patients are summarised in Table 5.7 (patients 1-5). Measurements of TBG, total T_3, TSH IRMA and dialysis fT_4 were normal in these patients, and no anti-T_4 or anti-T_3 antibodies were detected by the ammonium sulphate screening test (Section 2.3.1).

The serum from patient 1 was analysed by several free thyroid hormone kits (Table 5.8). Free T_4 values ranged from being slightly raised (SimulTRAC) to being greater than the top standard included with the kit. The Becton Dickinson fT_3 kit stood apart from the other fT_3 kits in giving a markedly elevated result. The interference caused by this serum, therefore, varied between different analogue RIA methods. Similar cases have been reported by others (Braverman & Ingbar, 1982; De Nayer *et al.*, 1984; Jackson & Ekins, 1986).

Table 5.8 Free Thyroid Hormone Concentrations by Several Analogue RIA Methods in Patient 1

Method	Reference Range (pmol/l)	Result (pmol/l)
Free T_4:		
Amerlex	10 - 22.5	38
Amerlex-M	10 - 21	45
Corning Magic	17 - 30	> 80
Becton Dickinson	8 - 17	>300
Coat A Count	11 - 23	48
SimulTRAC*	11 - 24	28
Free T_3:		
Amerlex	4.0 - 7.8	8.3
Amerlex-M	4.2 - 7.8	8.5
Becton Dickinson	5.3 - 10.0	20.0
Coat A Count	3.0 - 6.9	6.8

*SimulTRAC TSH = 3.8 mU/l (Ref. range: 0.9 - 6.7)

Table 5.7 Thyroid Function Tests in Patients with Altered Hormone-Binding to Albumin

Patient	Sex	Age (Yrs)	Total T_4 (nmol/l)	Total T_3 (nmol/l)	TBG (mg/l)	Albumin (g/l)	Amerlex fT_4 (pmol/l)	Amerlex fT_3 (pmol/l)	Boots-Celltech TSH IRMA (mU/l)	Dialysis fT_4 (pmol/l)	Dialysis fT_3 (pmol/l)
			Reference Range		M 9-31 F10-32	Ref.Range	Reference Range		Reference Range	Reference Range	
			60-145	1.1-2.7		36-47	10-22.5	4.0-7.8	0.14-5.9	8-17	3.2-6.7
1	M	28	187	2.6	20	53	38	8.3	3.2	13	6.0
2	F	84	181	1.4	25	40	42	4.1	2.3	15	4.6
3	F	38	227	1.8	27	45	50	5.1	2.5	10	2.7
4	F	42	174	2.2	31	47	33	5.5	0.2	17	3.9
5	F	59	190	2.1	-	45	38	7.6	2.3	15	6.0
6	F	30	130	8.3	32	45	25	28.8	3.8	12	4.6

Serum proteins from patients 1-5 were separated by electrophoresis after prior incubation with radiolabelled T_4 and T_3 to determine the distribution of thyroid hormone binding (Figure 5.8). There was significantly higher binding in the albumin zone and lower binding in the inter-alpha zone compared to normal sera. In this electro-phoretic system TBG is known to migrate in the inter-alpha zone (Beckett *et al.*, 1983b). The differences were more marked for T_4- rather than T_3-binding consistent with the greater distortion of total and free T_4 measurements in serum. Hennemann *et al.* (1982), Braverman & Ingbar (1982) and Stockigt *et al.* (1982b) have reported similar findings, and this abnormality is now known as familial dysalbuminemic hyperthyroxinemia (FDH). Patients with low TBG also had a shift of binding to albumin (Figure 5.8) but this was more marked, and the binding to TBG in the inter-alpha zone was substantially decreased. Serum from hyperthyroid patients showed a similar distribution of thyroid hormone binding to the euthyroid patients with FDH (Figure 5.8) and, therefore, dialysis free hormone measurements and TSH IRMA (or a TRH test) were necessary to distinguish the two situations.

Patient 6 (Table 5.7) had a normal total T_4 level but grossly elevated total T_3 and Amerlex fT_3 values. Concentrations of TSH IRMA and fT_3 measured by dialysis were normal in this patient. Electrophoresis showed

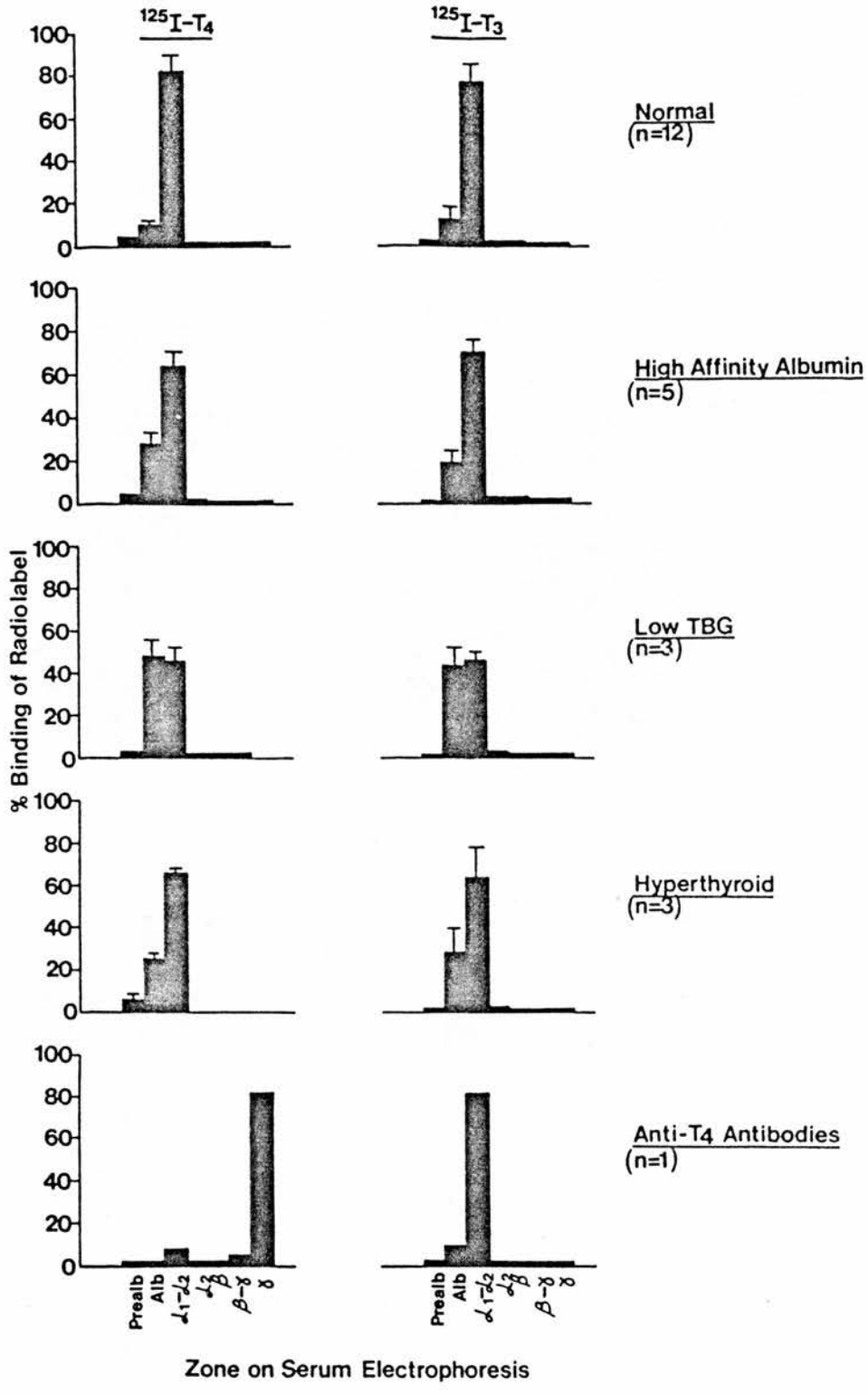


Figure 5.8 The Distribution of Thyroid Hormone Binding to Serum Proteins in Normal Sera and in Altered Protein-binding States.

a similar T_4-binding pattern to patients 1-5 above although there was slightly more binding of T_4 in the pre-albumin zone (8.1%). The distribution of T_3-binding, however, showed greater differences with 45% of T_3 bound in the albumin zone and 47% bound in the inter-alpha zone. This represents a rare case of FDH since in most cases only T_4-binding is significantly affected (Pearce & Byfield, 1986). The abnormality in FDH appears to be transmitted as an autosomal dominant trait (Hennemann et al., 1982) and there is some evidence that there is an increase in albumin with a particularly high affinity for T_4, normally present at a much lower concentration (Yabu et al., 1985).

5.2.3 Abnormal Thyroid Hormone-Binding to Anti-T_4 and Anti-T_3 Autoantibodies

Four patients who had total T_4 concentrations in serum which did not agree with the clinical impression were further investigated (Table 5.9). In patients 1-3 the clinical suspicion of hypothyroidism was confirmed by the raised TSH levels. Patient 1 had high titres of anti-microsomal (1:10,000) and anti-thyroglobulin (1:320) autoantibodies detected in his serum, patient 2 had been thyrotoxic 30 years previously and patient 3 was thought to have autoimmune hypothyroidism. Patients 1 and 2 have shown clinical improvement with T_4 replacement therapy.

Table 5.9 Patients with Increased Thyroid Hormone-Binding to γ-Globulin in Serum

Patient	Sex	Age (Yrs)	Boots-Celltech TSH IRMA (mU/l)	Total T_4 (nmol/l)	Total T_3 (nmol/l)	TBG (mg/l)	Amerlex fT_4 (pmol/l)	Amerlex fT_3 (pmol/l)	Dialysis fT_4 (pmol/l)	Dialysis fT_3 (pmol/l)	% γ globulin-binding on electrophoresis T_4	% γ globulin-binding on electrophoresis T_3
			Reference Range 0.14-5.9	Reference Range 60-145	1.1-2.7	M 9-31 F10-32	Reference Range 10-22.1	4.0-7.8	Reference Range 8-17	3.2-6.7	Reference Range <2%	<3%
1	M	29	39.0	168	0.4	28.0	>120	0.9	5	1.3	87	2
2	F	64	20.0	131	1.2	31.0	>120	4.2	6	4.0	18	3
3	F	69	16.9	188	1.4	25.0	>120	3.6	8	5.8	9	1
4	F	88	4.3	286	0.6	16.5	14	1.0	13	1.7	6	2

Patient 4 had been commenced on carbimazole on the basis of the raised total T_4 level but this was stopped when total T_4 levels remained persistently raised and there was no clinical improvement.

All of these patients had normal concentrations of IgG, IgA and IgM in their serum, and total and fT_3 concentrations were either normal or low. Free T_4 by Amerlex RIA was grossly elevated in all but patient 4. A raised fT_4 value (>70 pmol/l) was also found for patient 3 by SimulTRAC assay. Free T_4 values by dialysis-RIA were low or borderline-low in those with raised TSH levels. Sera from all of these patients gave a positive result for T_4-autoantibodies in the screening test and an increased binding to T_4 in the γ-globulin region on electrophoresis (Table 5.9). This was particularly evident for patient 1 (Figure 5.8). The affinity and binding capacity of the IgG T_4-autoantibody identified in this patient's serum was characterised by Archbold *et al.* (1986) as having a K_a of 1.2×10^8 l/mol and a concentration of 0.3 µmol/l giving a binding capacity approximately equivalent to that of TBG.

The normal Amerlex fT_4 result obtained for patient 4 was supported by the fact that when the Amerlex ^{125}I-T_4 analogue was used in the autoantibody screening test rather than ^{125}I-T_4, a reduction in binding to 6% compared to 3% for control sera was observed. In this

case, the anti-T_4 antibodies did not appear to recognise the analogue used in this fT_4 kit, in contrast to the other 3 patients where interference was much greater in the analogue fT_4 assay compared to the total T_4 assay. A similar case was described by Byfield et al. (1983).

Autoantibodies to T_3 have also been described which cause spuriously abnormal results for total T_3 and high fT_3 results by analogue RIA (Staeheli et al., 1975; Byfield et al., 1983; Beckett et al., 1983b; Rodriguez-Espinosa et al., 1984). Abnormal antibody-binding is commonly reported in patients with autoimmune thyroid disorders (Beck-Peccoz et al., 1982) and it is suggested that thyroglobulin may act as the immunogen (Pearce et al., 1981). The prevalence on routine screening however, appears to be quite low i.e. 0.05% (Fielding, 1984).

5.2.4 Summary

Alterations in the binding of thyroid hormones to serum protein may occur due to (a) alterations in the concentration of binding proteins, particularly TBG (b) alterations in their affinity e.g. FDH and (c) the presence of abnormal thyroid hormone-binding antibodies. Concentrations of total and free thyroid hormones by analogue-RIA may deviate from normal in euthyroid patients with these alterations and diagnostic confusion may arise where there is underlying thyroid disease. In theory,

measurements of free thyroid hormone concentrations should reflect the true thyroid status in such patients but, in practice, the presence of high affinity albumin, TBPA (Skiest et al., 1986) or autoantibodies to T_4 and/or T_3 interfere with the analogue free hormone assays. Measurements by equilibrium dialysis provide an accurate measure of thyroid hormone status in these patients. Methods which use microencapsulated antibodies, two-step back titration or column adsorption chromatography also appear to give accurate results (Beck-Peccoz et al., 1982; Shah & Shah, 1984; Jackson & Ekins, 1986; Skiest et al., 1986; Archbold et al., 1986). However, measurement of TSH by IRMA clearly identified those patients with thyroid dysfunction from euthyroid patients who had altered hormone-binding and who did not require treatment. Basal TSH (IRMA), therefore, represents the routine test of choice in such patients.

Chapter 6

THE ASSESSMENT OF THYROID STATUS IN PATIENTS WITH NON-THYROIDAL ILLNESS

In this chapter, the results of thyroid function tests in patients with non-thyroidal illness are presented. Firstly, the test specificities in patients from a general medical ward and in geriatric patients are investigated followed by more detailed study of patients with chronic obstructive airways disease and patients with chronic renal failure.

6.1 THYROID FUNCTION TESTS IN PATIENTS FROM A GENERAL MEDICAL WARD

6.1.1 The Age, Sex and Disease Distributions of the Study Group

The patients studied were 264 consecutive admissions to a general medical ward (137 males, 127 females) excluding those with a past history or clear clinical evidence of thyroid disease. These patients were older than the 97 euthyroid patients from the thyroid clinic (Section 4.1) and their ages showed a skewed distribution (range 16-96, mode 75 years; Figure 6.1). Cardio/cerebrovascular, respiratory, liver and gastro-intestinal problems were the reason for admission in 40%, 19%, 9% and 8%, respectively. The remaining 24% of patients were admitted with a variety of other illnesses. Most patients were taking a variety of drugs as therapy for chronic ailments or for treatment of their acute symptoms. Serum samples were taken on the day of admission and stored at -20°C until analysis.

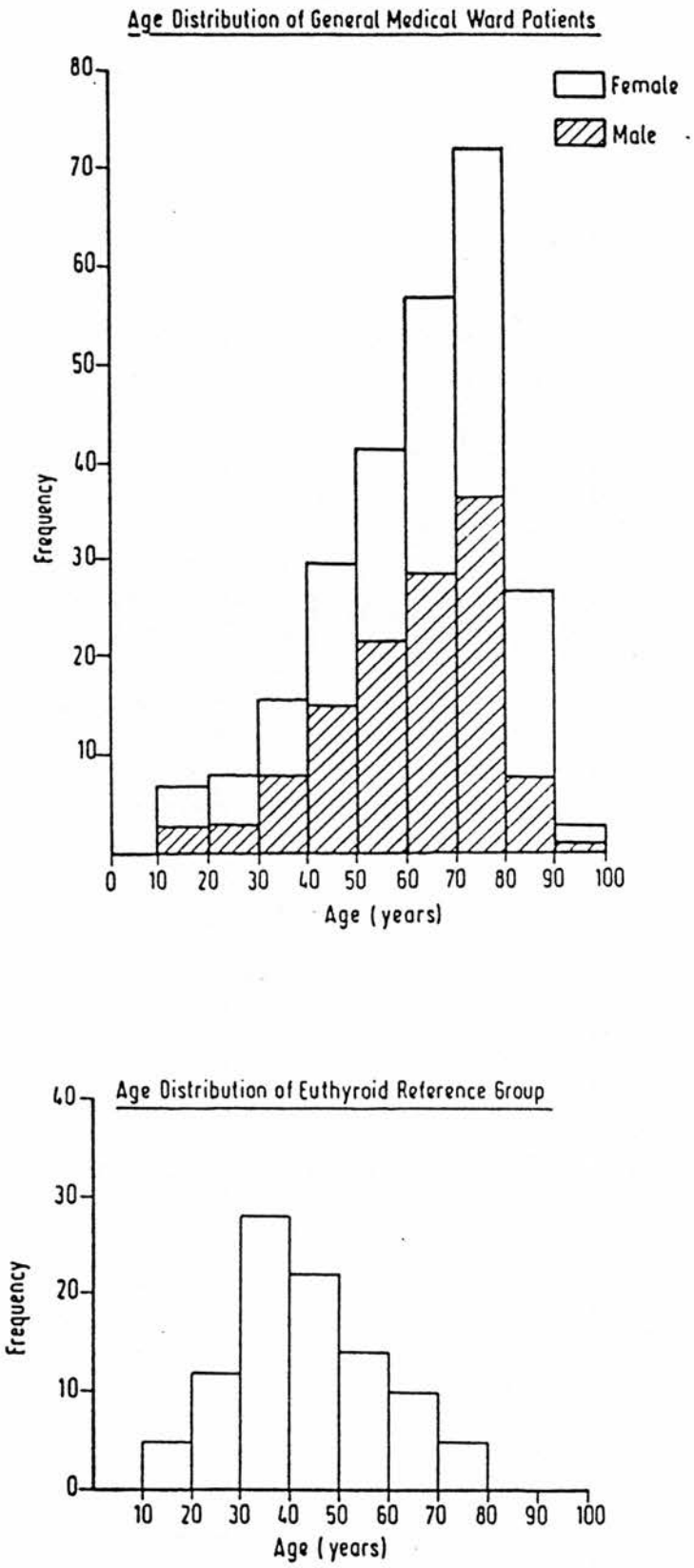


Figure 6.1 Age Distributions of Patients in a General Medical Ward and the Reference Group.

6.1.2 Results for Thyroid Function Tests

The number of patients with abnormal results for total T_4, fT_4 (Amerlex), total T_3, fT_3 (Becton Dickinson) and TSH IRMA (Boots-Celltech) in serum are shown in Table 6.1. The reference ranges used were: total T_4 60-145 nmol/l, fT_4 10.0-22.5 pmol/l, total T_3 1.0-2.7 nmol/l, fT_3 5.3-10.0 pmol/l and TSH IRMA 0.14-5.9 mU/l. These were derived from the results of 97 euthyroid patients (Section 4.6), and now used as the reference group.

Table 6.1 The Number of Abnormal Thyroid Function Tests in 264 Patients from a General Medical Ward

	Result	
Test	Low	High
Total T_4	21 (8%)	3 (1%)
fT_4	40 (15%)	2 (<1%)
Total T_3	50 (19%)	0 -
fT_3	128 (49%)	2 (<1%)
TSH IRMA	3* (1%)	15 (6%)

*Undetectable

A large proportion (49%) of patients had low fT_3 values. This test, therefore, lacked specificity for thyroid disease in this group. In contrast to the other tests which showed no dependence on age, patients with low total T_3 concentrations were significantly older than those with normal total T_3 levels ($p<0.01$, χ^2 test). Results for total T_4, fT_4 and TSH IRMA are shown in Figure 6.2.

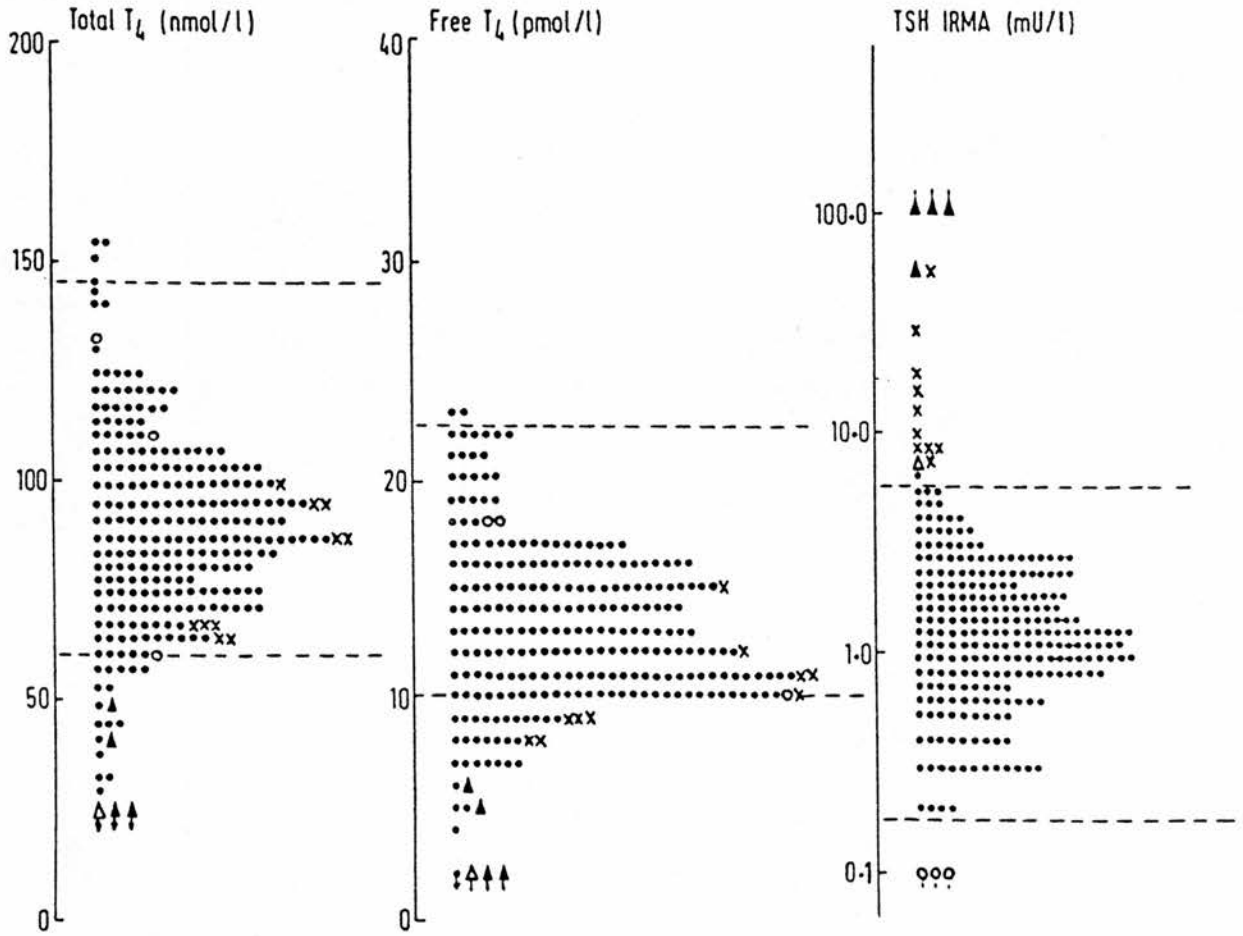


Figure 6.2 Serum Total T_4, Free T_4 (Amerlex) and Basal TSH IRMA in Patients from a General Medical Ward.

Results for patients with undetectable TSH IRMA (o), overt hypothyroidism (▲) and elevated TSH IRMA but normal total T_4 (x), are as indicated. One patient (Δ) had septicaemia and later died.

(a) Patients with Low Total T_4 and Raised TSH Levels

Eighteen patients had low total T_4 and low fT_4 concentrations but only four of these had unequivocally high TSH concentrations who, on recall, showed clinical evidence of hypothyroidism; these patients have since commenced T_4 replacement therapy. Two of these patients had other diseases known to be associated with primary hypothyroidism: one had pernicious anaemia and the other had haemochromatosis. One other patient, who died soon after admission, had low thyroid hormone concentrations and a TSH IRMA result of 7.4 mU/l.

(b) Patients with Low Total T_4 and Normal TSH Levels

Details of the 16 patients in this category are given in Table 6.2. The majority also had low results for fT_4, total T_3 and fT_3. Results for TSH were not clustered towards the upper reference limit and in fact were significantly lower than those in the reference group ($p<0.05$, Mann-Whitney test). Four patients were drug-free, those with epilepsy were taking phenytoin or sodium valproate and the remainder were receiving digoxin, diuretics or antibiotics. Serum albumin and total protein concentrations were low in 9 and 5 of those patients tested, respectively.

(c) Patients with Low Free T_4, Normal Total T_4 and Normal TSH Levels

In addition to the 13 patients with low fT_4 described in Table 6.2, 18 further patients had low

Table 6.2 Patients with Low Total T_4 and Normal TSH Concentrations

Patient	Sex	Age	Main Diagnosis	Drugs	TSH IRMA (mU/l)	T_4 (nmol/l)	fT_4* (pmol/l)	T_3 (nmol/l)	fT_3† (pmol/l)	TP (g/l)	ALB (g/l)
1	M	59	Hepatic cirrhosis	None	0.25	58	10.4	1.0	3.5	77	35
2	F	82	Varicose ulceration	None	0.9	44	6.5	<0.5	<1.0	57	28
3	F	70	Biventricular failure	Bendrofluazide	2.1	43	7.1	<0.5	<1.0	62	35
4	F	16	Epileptic fit	Sodium valproate	1.2	52	9.7	1.3	5.8	65	39
5	M	68	Femoral embolus Acute renal failure	Digoxin, Isosorbide	0.5	32	5.0	0.4	<1.0	63	35
6	F	29	Epileptic fit	Phenytoin, Phenobarbitone	0.3	48	7.9	1.2	5.6	62	38
7	F	68	Pneumonia	Digoxin, Tenoretic	1.3	59	6.8	0.6	<1.0	62	30
8	F	53	Halothane hepatitis	Cefuroxime, Metronidazole	0.6	38	5.0	0.2	<1.0	55	21
9	F	90	Diabetic ketoacidosis	Burinex, Insulin, Ampicillin	0.5	58	11.1	0.7	3.0	58	36
10	M	72	Chest infection	Tetracycline, Diazepam	1.0	49	6.8	1.0	3.4	ND	32
11	F	62	Ulcerative colitis	None	1.2	32	<0.5	<0.5	<1.0	42	19
12	M	65	Hyperosmolar non-ketotic hyperglycaemia	Nifedipine, Frusemide, Spironolactone	0.3	57	8.6	0.9	3.5	ND	36
13	F	34	Nephrotic syndrome	Frusemide	5.0	29	4.1	0.7	1.4	33	13
14	M	59	Chronic bronchitis	NA	0.8	44	8.8	0.9	2.6	ND	30
15	M	78	Alcoholic liver disease	NA	0.8	55	16.9	0.7	4.2	ND	39
16	F	70	Diabetes	NA	4.2	44	8.8	0.5	1.0	ND	34

Results below the reference range are underlined. Laboratory reference ranges for total protein (TP) and albumin (ALB) were 60-80 and 36-47 g/l, respectively. *Amerlex fT_4, †Becton Dickinson fT_3, ND = not determined.
NA = Information not available.

fT4 with normal total T4 and TSH, Table 6.3. Thyrotrophin results in patients with low fT4 were not significantly different from the reference group. Serum albumin and total protein concentrations were below reference limits in 48% and 23% of these patients, respectively.

(d) Patients with Raised Concentrations of Thyroid Hormones

A small number of patients had slight elevations in thyroid hormone concentrations; TSH IRMA was detectable in all of these patients (Table 6.4). In two patients, the high total T4 levels could be accounted for by oestrogen-induced TBG synthesis: patient 1 was taking an OCP and patient 2 was taking Tamoxifen, a drug known to increase ovarian oestrogen synthesis by decreased feedback inhibition of the pituitary (Fex et al., 1981). A small number of patients (2.5%) would also be expected to have raised concentrations since the reference range was calculated to include only 95% of euthyroid values.

(e) Patients with Raised TSH and Normal T4 Levels

Ten patients (8 females, 2 males; aged 56-86 yrs) had TSH values greater than 7.0 mU/l but normal total T4 concentrations; fT4 concentrations were low in 5 of these patients (Figure 6.2 and Table 6.5). These low fT4 values could not be attributed to low serum albumin concentrations or drugs known to lower Amerlex fT4 values. One patient (patient 3) had repeat tests three months after the first admission to hospital; his TSH concentra-

Table 6.3 Patients with Low Free T_4, Normal Total T_4 and Normal TSH Concentrations

Patient	Sex	Age	Main Diagnosis	Drugs	TSH IRMA (mU/l)	T_4 (nmol/l)	*fT_4 (pmol/l)	T_3 (nmol/l)	†fT_3 (pmol/l)	TP (g/l)	ALB (g/l)
1	F	67	Chronic bronchitis	Salazopyrin, Ampicillin, Prednisolone, Flurazepam	0.8	65	7.1	1.1	3.9	66	40
2	F	42	Crohn's disease	None	1.6	69	9.7	1.0	2.8	53	31
3	M	47	Chronic bronchitis	None	0.7	71	9.7	1.6	6.1	72	39
4	M	39	Pulmonary embolus	Metoprolol	0.6	87	9.0	1.8	7.8	66	42
5	M	60	Chronic bronchitis/ collapse	None	0.8	108	8.5	1.7	7.8	70	46
6	F	35	Pulmonary embolus	None	3.4	88	8.3	1.8	6.2	66	37
7	M	55	Gastric ulcer	Anadin	0.7	68	6.8	1.1	2.5	77	38
8	F	52	Falls	Paracodol	1.4	67	9.0	1.1	4.4	77	32
9	F	36	Acute glomerulonephritis	Triamterene	1.3	84	8.3	1.4	4.8	55	29
10	M	70	Deep vein thrombosis	Digoxin, Navidrex K	3.0	87	9.7	1.5	5.7	65	38
11	M	68	Pneumonia	None	2.6	61	8.2	0.8	1.7	66	30
12	M	43	Angina	None	1.5	63	9.5	1.6	4.6	56	34
13	F	72	?Angina	Mefanamic acid, Aludrox	3.4	90	9.5	2.1	6.0	69	43
14	F	79	Cholangiocarcinoma	Cholestyramine	0.9	76	8.0	0.7	<1.0	ND	30
15	M	59	Metastatic adenocarcinoma	None	3.9	97	8.1	1.3	2.8	ND	35
16	F	60	Epileptic fit	Phenytoin, Phenobarbitone	6.4	73	9.5	1.6	1.1	ND	39
17	F	53	Diabetic ketoacidosis	Insulin	5.6	63	7.3	1.2	4.7	ND	36
18	F	77	Epilepsy	NA	2.0	60	9.4	1.2	5.1	ND	36

Results below the reference range are underlined. TP = Total Protein, ALB = albumin, ND = Not determined, NA = Information not available, *Amerlex fT_4, †Becton Dickinson fT_3.

Table 6.4 Patients with Raised Thyroid Hormone Concentrations

Patient	Sex	Age	Main Diagnosis	Drugs	TSH IRMA (mU/l)	T_4 (nmol/l)	fT_4* (pmol/l)	T_3 (nmol/l)	fT_3† (pmol/l)	TP (g/l)	ALB (g/l)
1	F	24	Crohn's disease	Prednisolone, Salazopyrin, OCP	0.5	153	14.6	1.6	3.7	80	44
2	F	33	Pulmonary embolus	Warfarin, Tamoxifen	0.9	152	15.7	1.2	1.6	62	40
3	M	31	Peptic ulcer	-	4.1	151	24.0	1.1	4.9	ND	ND
4	M	64	Angina	Ipratropium, Salbutamol, Feldene	1.1	146	23.0	2.2	8.1	72	37
5	M	18	Pneumonia	Salbutamol	3.2	113	21.0	2.2	11.5	71	48
6	M	43	Angina	None	2.4	105	21.0	2.2	11.8	78	46

Results above the reference range are underlined. ND = Not determined, TP = Total Protein, ALB = Albumin.
*Amerlex fT_4, †Becton Dickinson fT_3.

Table 6.5 Patients with Raised TSH and Normal Total T_4 Concentrations

Patient	Sex	Age	Main Diagnosis	Drugs	TSH IRMA (mU/l)	T_4 (nmol/l)	fT_4* (pmol/l)	T_3 (nmol/l)	fT_3† (pmol/l)	TP (g/l)	ALB (g/l)
1**	F	82	Myocardial infarction	Burinex K, Digoxin	7.6	96	11.0	1.7	6.0	75	40
2	F	67	Myocardial infarction	None	29.0	88	9.3	2.1	7.5	76	43
3**	M	72	Left ventricular failure	Metformin, Chlorpropamide	16.0	67	9.5	1.2	5.8	66	40
4	F	56	Post cholecystectomy pain	None	8.6	88	11.6	2.2	9.6	78	46
5	F	62	Angina	Burinex K, Nifedipine, Amitryptiline, Isosorbide	55.0	69	10.5	1.3	6.7	77	43
6	F	67	Angina	Glyceryltrinitrate	8.8	93	7.7	1.9	6.7	ND	43
7**	F	86	Cerebrovascular accident	None	12.6	64	8.1	1.4	5.5	ND	42
8	M	72	Cerebrovascular accident	Navidrex K, Aspirin, Salbutamol	18.1	62	8.6	1.1	3.5	ND	37
9	F	66	Severe chronic bronchitis	NA	8.8	87	11.0	1.4	6.0	ND	ND
10	F	80	Small myocardial infarction	NA	8.9	68	16.0	0.8	6.2	ND	ND

Results consistent with hypothyroidism are underlined. Patients 1,2,4,6 had positive anti-thyroid autoantibody tests.
**Patient died. TP = Total Protein, ALB = Albumin; ND = Not determined, NA = Information not available.
*Amerlex fT_4, †Becton Dickinson fT_3.

tion remained elevated at 23 mU/l although antithyroid autoantibodies were not detected. This patient has since died. Positive anti-thyroid microsomal titres were found in 4 of the other patients' sera. Unfortunately, two other patients died soon after their hospital admission and the rest were lost to follow-up. The presence of mild hypothyroidism could not, therefore, be firmly established by follow-up study.

(f) Patients with Undetectable TSH IRMA Levels

Three patients with no clinical evidence of hyperthyroidism at admission had undetectable basal TSH concentrations by IRMA (Figure 6.2). Details of these patients are given in Table 6.6 (patients 1-3). One patient had borderline-high total and free T_4 concentrations on later testing by her general practitioner, consistent with subclinical hyperthyroidism; the remaining two patients were lost to follow-up. Also shown in Table 6.6 are results for two patients (patients 4 and 5) who were excluded from the study because of the finding of a large multinodular goitre at examination.

In summary, many abnormal thyroid function test results were identified in this series of patients raising the question as to which measurement most accurately reflects thyroid status in the presence of coexistent illness. It was thought that a true measure of free thyroid hormone concentrations by equilibrium dialysis

Table 6.6 Patients with Undetectable TSH IRMA in Serum

Patient	Sex	Age	Main Diagnosis	Drugs	TSH IRMA (mU/l)	T_4 (nmol/l)	fT_4* (pmol/l)	T_3 (nmol/l)	fT_3† (pmol/l)	TP (g/l)	ALB (g/l)
1**	F	69	Cerebrovascular accident	Aspirin	<0.1	132	18.0	2.1	9.5	ND	44
2	F	82	Wedge fracture	Bendrofluazide, Paracetamol	<0.1	110	18.0	1.8	7.9	64	41
3	M	33	Alcohol withdrawal fit	None	<0.1	60	9.8	1.0	5.1	ND	42
4	F	72	Granulomatous hepatitis	Patients excluded due to large multi-nodular goitres	<0.1	151	24.6	1.2	2.7	ND	33
5	M	80	Deep vein thrombosis		<0.1	108	15.0	1.8	6.1	ND	38

Results consistent with hyperthyroidism are underlined. *Amerlex fT_4, †Becton Dickinson fT_3,
**Borderline-high thyroid hormone results on later testing. TP = Total Protein, ALB = Albumin, ND = Not determined.

might help answer this question since the influence of changes in serum albumin concentration or drug effects on values by analogue RIA may be substantial in these patients, as discussed below.

6.1.3. The Effects of Non-thyroidal Illness on Free Thyroid Hormone Measurements

(a) Results by Analogue RIA and Equilibrium Dialysis

Results for fT_4 and fT_3 by several methods are shown in Figure 6.3 for the first 40 patients described in Section 6.1. The Corning Magic, Becton Dickinson and Coat A Count kits produced most fT_4 values below reference limits (6 or 7 patients). Dialysis of serum (1:20 dilution) revealed five patients with low fT_4 of whom four had low values by all of the analogue RIA methods. Three patients had raised values by dialysis but normal results by analogue RIA. One of these patients had an absent TSH response to TRH but there were no other test results to suggest hyperthyroidism and she died shortly after admission to hospital with a chest infection (see later: Table 6.9, patient 1).

A greater proportion of patients had low fT_3 values by analogue assay ranging from 14 to 18 patients depending on the kit (Figure 6.3). Dialysis of serum (1:10 dilution) showed only six patients with fT_3 values below reference limits. However, fT_3 values by dialysis in these 40 sera (mean, 4.5 ± 1.2 pmol/1) were significantly lower ($p<0.05$) than those in sera from euthyroid

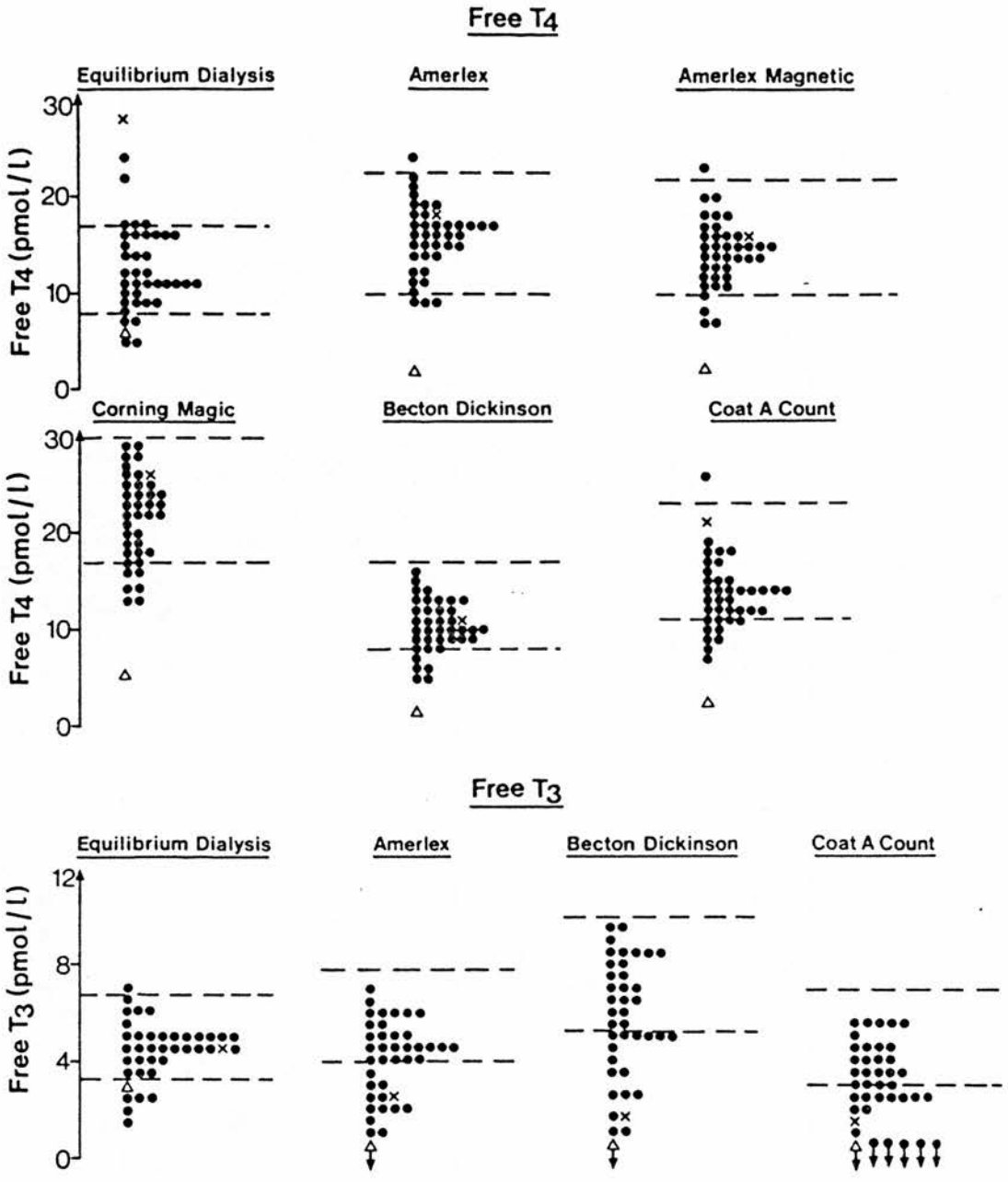


Figure 6.3 Concentrations of Free T_4 and Free T_3 by Different Methods in 40 Patients with Non-thyroidal Illness.

One patient (Δ) had an elevated TSH concentration and one patient (x) had an absent TSH response to TRH.

outpatients (n=15) analysed in the same dialysis runs (mean, 5.2±0.6 pmol/l).

(b) Serum Albumin and Free Thyroid Hormone Results

The dependence of fT_4 and fT_3 results by analogue assay on the serum albumin concentration was demonstrated previously (Section 5.1.5) by the *in vitro* addition of human albumin to serum. Measurements by dialysis were not affected by added albumin. In the medical in-patients a greater range of albumin concentrations in serum was present compared to the reference group of euthyroid out-patients, and positive correlations with fT_4 (Amerlex) and fT_3 (Becton Dickinson) were stronger (Figures 6.4 and 6.5).

In the 40 sera with dialysis values for free thyroid hormones, serum albumin ranged from 30-48 g/l (mean 40.6±4.5 g/l) and, unlike analogue-RIA values, dialysis fT_4 and fT_3 values did not correlate significantly with serum albumin (Table 6.7). Indeed, fT_3 values by analogue-RIA correlated more strongly with serum albumin than with dialysis values for fT_3.

This and several other reports (Bayer 1983a; Liewendahl *et al.*, 1984; Ooi & Sorisky, 1984; Csako *et al.*, 1986) have confirmed the albumin-dependence of analogue RIA measurements reported originally by Stockigt *et al.*, in 1981. In spite of reformulations by various manufacturers, this problem has still to be resolved completely (Wilkins *et al.*, 1985; Beck *et al.*, 1987).

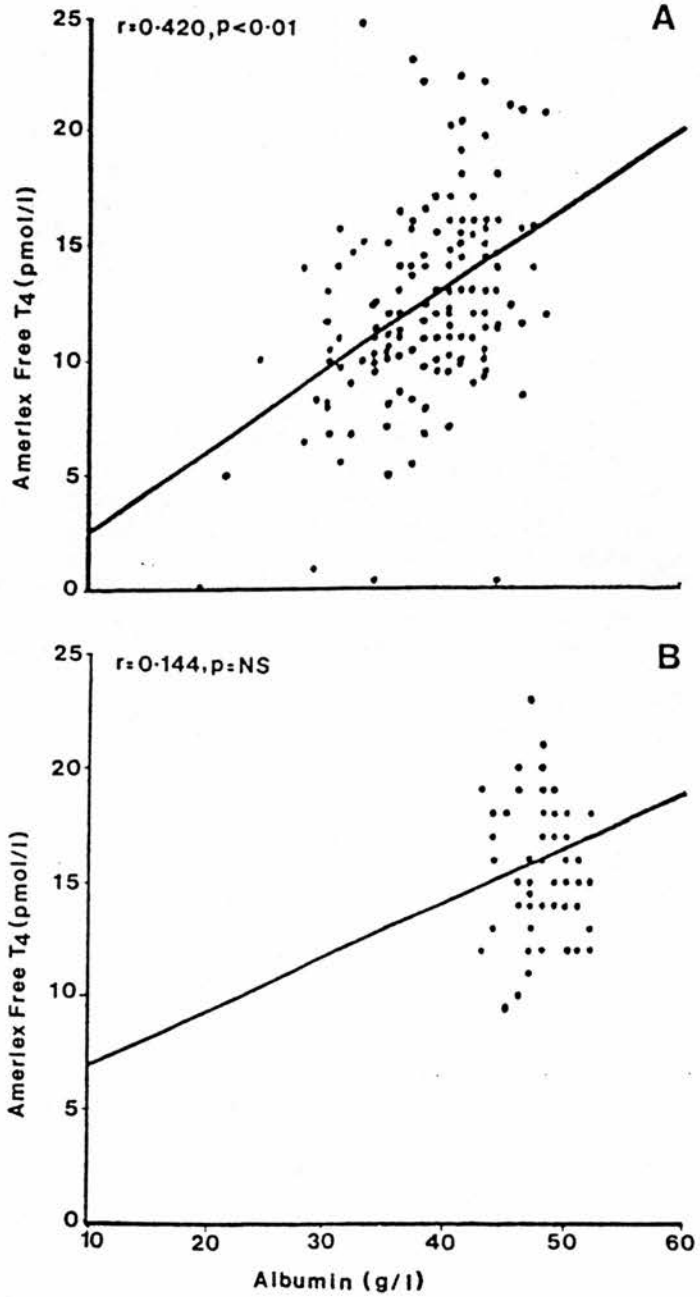


Figure 6.4 Correlations between Free T_4 (Amerlex) and Serum Albumin.

(A) General medical in-patients.
(B) The reference group of euthyroid out-patients.

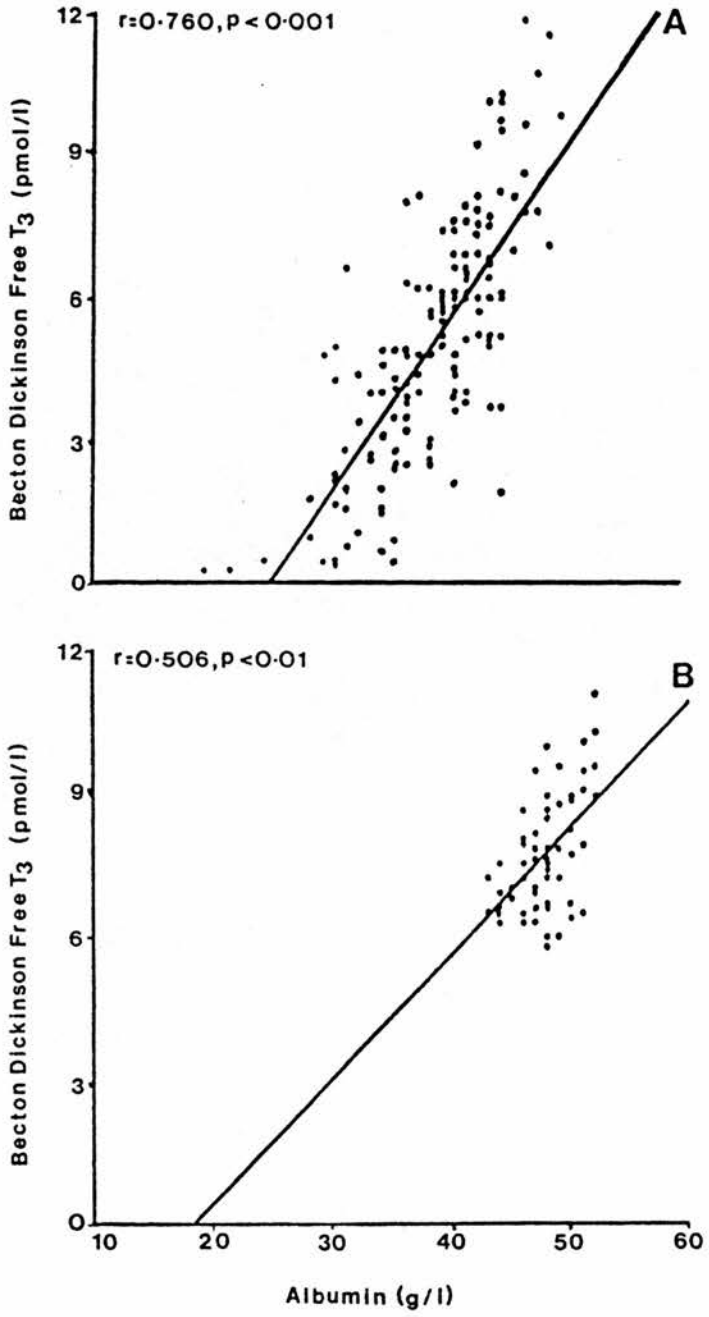


Figure 6.5 Correlations between Free T_3 (Becton Dickinson) and Serum Albumin.

(A) General medical in-patients.
(B) The reference group of euthyroid patients.

Table 6.7 Correlations Between Analogue Free Thyroid Hormone Levels, Dialysis Values, Albumin and NEFA Concentrations in Non-thyroidal Illness

	Correlations(r) with:-		
	Dialysis Values	Albumin	NEFA
Free T_4 Method			
Dialysis	-	0.132	-0.225
Amerlex	0.539[a]	0.424[b]	-0.030
Amerlex-M	0.542[a]	0.455[b]	0.027
Corning Magic	0.522[a]	0.484[b]	-0.093
Becton Dickinson	0.500[b]	0.504[b]	-0.004
Coat A Count	0.542[a]	0.275	-0.163
Free T_3 Method			
Dialysis	-	0.313	-0.494[b]
Amerlex	0.546[a]	0.826[a]	-0.297
Becton Dickinson	0.479[b]	0.887[a]	-0.248
Coat A Count	0.575[a]	0.772[a]	-0.391

a) $p<0.001$, b) $p<0.01$

Significant correlations with serum albumin were also observed for total T_3 ($r=0.598$, $p<0.001$) and total T_4 ($r=0.262$, $p<0.05$) in patients from the medical ward (Section 6.1, $n=160$) but these were of lower order than those for fT_3 and fT_4 by analogue RIA. Such correlations with total thyroid hormone concentrations would be anticipated since patients with low serum albumin concentrations might also have low TBG concentrations due to generalised protein loss or decreased protein synthesis. Values for TSH IRMA did not correlate significantly with albumin concentrations.

(c) The Effect of Increased NEFA Concentrations in Serum

Heparin administration activates lipoprotein lipase in the endothelial lining of blood vessels leading to release of NEFA (Riemersma et al., 1981) resulting in high fT_4 levels measured by dialysis but low results by analogue RIA (Wilkins et al., 1982; Ekins et al., 1983; Bayer, 1983b). A similar discrepancy exists in patients with non-thyroidal illness (NTI) who may be catabolic with high levels of circulating NEFA due to mobilisation of fat from adipose tissue. Since NEFA bind to albumin in blood, they may displace thyroid hormones leading to high free hormone levels. The significance of the low results by analogue assay remains unclear.

(i) In vitro effects

The effect of exogenous NEFA on the measurement of fT_4 and fT_3 in serum was investigated by the addition of oleic acid (Sigma) to aliquots of two serum pools to produce an increase in NEFA of 0.2 - 30.0 mmol/l with minimal (<1%) dilution of the pools. Results by dialysis -RIA and the Amerlex methods are shown in Figure 6.6. The effects of added oleic acid on fT_4 were much greater than for fT_3 by both methods. There was little change in Amerlex fT_4 values until the NEFA concentration increased by 2 mmol/l, and at 5 mmol/l they had halved, which confirms the findings of Wilkins et al., 1982 & 1985. At NEFA levels >10 mmol/l, Amerlex fT_4 values then increased

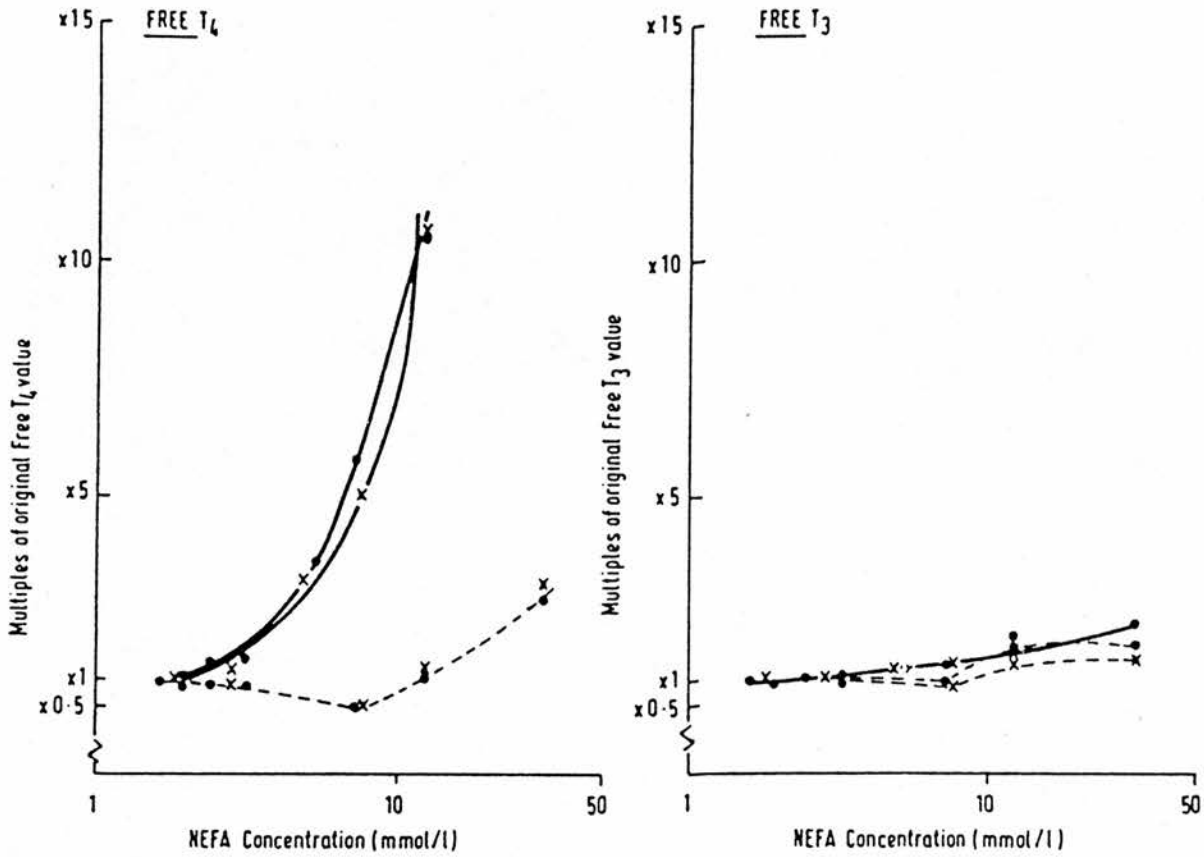


Figure 6.6 The Effect of Added Oleic Acid on Free Thyroid Hormone Measurement in Two Serum Pools (● and x).

Solid lines are equilibrium dialysis values. Dashed lines are Amerlex values.

rapidly. By equilibrium dialysis, fT_4 values increased exponentially and had risen by 80-100% for an increase of 2 mmol/l NEFA; there was little change in fT_3 levels until the NEFA concentration exceeded 3 mmol/l, in agreement with the early study of Hollander *et al.*, 1967. Wilkins *et al.* in 1986 recently confirmed the relative insensitivity of the Amerlex fT_3 method to added oleic acid but found greater increases in fT_3 than those shown in Figure 6.6 for equilibrium dialysis and Amerlex methods when NEFA levels exceeded 2 and 6 mmol/l, respectively.

(ii) *In vivo effects*

Confirmation that high NEFA levels could affect fT_4 values *in vivo* was obtained by studying patients undergoing coronary artery by-pass surgery where mobilisation of fat stores occurs due to the fasting and stress of surgery; some heparin anticoagulation is also given. Dilution profiles for serum fT_4 by equilibrium dialysis are shown in Figure 6.7(a) for six patients post by-pass operation. Concentrations of NEFA were markedly raised (1-4 mmol/l) and an increase in fT_4 values of 50-100% above normal would be predicted from the *in vitro* effect observed with oleic acid addition (Figure 6.6). The rank order of dialysis fT_4 values *in vivo* was not exactly the same as that for the NEFA concentrations (Figure 6.7(a)), but the dilution profiles were markedly different from those shown for sera from an out-patient thyroid clinic

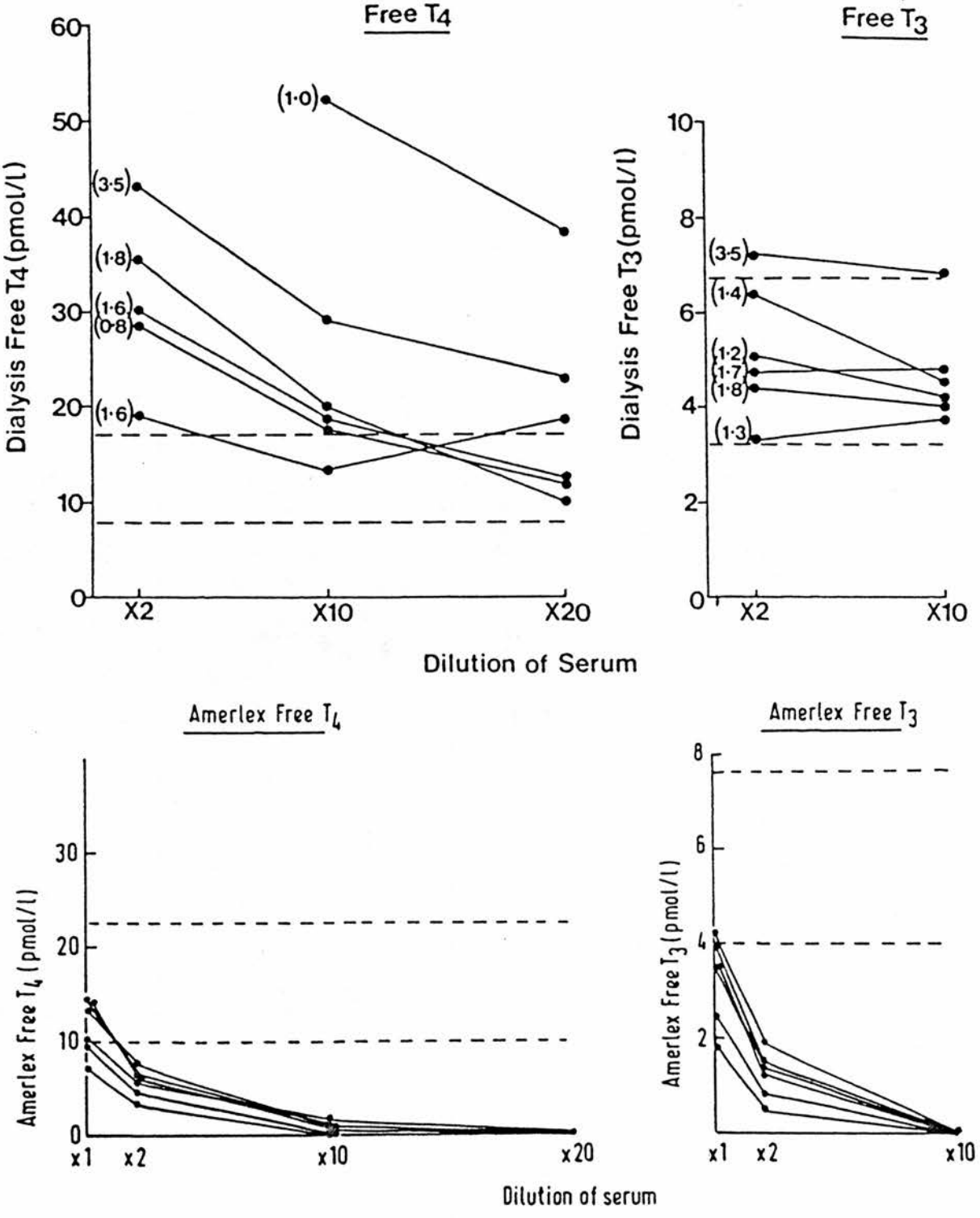


Figure 6.7 The Effect of Serum Dilution prior to the Assay by Equilibrium dialysis (a) and Amerlex RIA (b) of Free T_4 and T_3 in Patients with High Concentrations of NEFA in Serum.

In (a) NEFA concentrations for each serum are shown in brackets.

(Figure 3.14). In the by-pass patients, fT_4 values were raised at low dilutions of serum and these decreased progressively with dilution of serum prior to dialysis. At a 1:20 dilution of serum, fT_4 values exceeded the upper reference limit in half of the patients studied. Values for fT_3 decreased by only 10% comparing 1:2 and 1:10 dilutions of serum (Figure 6.7(a)), and high values were found in only 1 of the 6 sera studied. This is consistent with the smaller effect of added oleic acid on serum fT_3 found *in vitro*, and supports the results of an earlier study of the *in vivo* effects of heparin administration on dialysis fT_3 values (Thomson *et al.*, 1977).

Dilution of these sera prior to Amerlex RIA resulted in a dramatic fall in fT_4 and fT_3 values (Figure 6.7(b)). This has been reported as a general observation for all patients' sera (Wilke, 1982; Swift & Ratcliffe, 1983) and has raised the question of the validity of these tests. It has been argued, however, that the dilution test is not an appropriate test of the validity of free hormone measurements by analogue RIA because the use of an antibody as a sampling device "narrows the dilution range over which fT_4 remains constant" (Wilkins, 1986). Aside from this issue, it is clear that in these patients, values by analogue RIA tended to be low, in agreement with the findings of Bayer (1983b) in heparin-treated patients.

In three patients, dilution profiles for fT_4 by dialysis were performed before and after by-pass surgery (Table 6.8). The fT_4 dilution profiles were most affected in sera taken after the operation, when NEFA concentrations were highest.

Table 6.8 Dialysis Free T_4 Values Pre- and Post-By-pass Surgery

Time after surgery (h)	NEFA (mmol/l)	Dialysis Free T_4 (pmol/l) Serum Dilution 1:2	1:5	1:10	1:20
Patient 1					
0	0.69	8.6	7.2	6.3	5.0
6	0.92	13.0	8.3	7.3	6.7
24	2.31	15.7	12.5	8.7	10.8
Patient 2					
0	0.76	-	10.9	9.8	10.3
6	1.24	19.8	18.7	18.3	17.5
24	1.76	26.5	24.0	17.3	16.1
Patient 3					
0	0.53	-	15.3	14.8	16.6
6	1.36	-	54.4	30.0	24.8

In the medical ward, NEFA concentrations would not, in general, be as high as in heparin-anticoagulated patients post-surgery and, therefore, values for fT_4 by dialysis would be less affected by serum dilution (unless other inhibitors of T_4-binding were also present). In the 40 patients (Figure 6.3) where fT_4 was measured by dialysis using serum diluted 1:20, NEFA values ranged from 0.2-2.2 mmol/l (mean, 0.74±0.49). Free T_4 values by both

dialysis and analogue RIA did not correlate significantly with NEFA concentrations (Table 6.7),and in those with the highest dialysis fT_4 values (28,24,22 pmol/l), NEFA levels were 1.3, 0.2 and 2.2 mmol/l, respectively.

Paradoxically, fT_3 dialysis values showed an inverse correlation with NEFA levels *in vivo* (Table 6.7) as did total T_3 values but this was less significant than with fT_3 by dialysis, $r = -0.363$ ($p<0.05$). In fasting individuals, an increase in the peripheral conversion of T_4 to rT_3 is known to occur resulting in low total T_3 concentrations (Braverman & Vagenakis, 1979) and in prolonged fasting, the breakdown of lipid from adipose tissue to NEFA occurs. The correlation observed here between dialysis fT_3 and NEFA concentrations may reflect the change, in opposite directions, of these analytes as a result of poor nutritional status.

In patients with co-existent illness, free thyroid hormone status is difficult to assess by analogue-RIA due to the low albumin concentrations in serum of many patients. Fewer patients have low values by dialysis-RIA but an increase in values, particularly for fT_4, occurs in situations where concentrations of NEFA or other inhibitors of T_4-binding in serum are high.

6.1.4 The Relationship Between Basal TSH IRMA and the TSH Response to TRH in Non-thyroidal Illness

Seventy-four patients (47 males, 27 females; mean age 66 years) from the medical ward had a TRH test performed

in which serum TSH was measured by in-house RIA. The good correlation between basal TSH (IRMA) and the TSH increment 20 min after TRH found in euthyroid out-patients was also present in patients with NTI ($r=0.648$, $p<0.001$), although the scatter of points was greater (Figure 6.8). Basal TSH (RIA) and the response to TRH were significantly higher in female compared to male patients (Figure 6.9).

Diminished TSH (RIA) responses to TRH (less than 3.9 mU/l TSH increment) were found in 30 patients and predominantly in the older men (Mann-Whitney test, $p<0.05$). Total and free T_4 concentrations in those with reduced responsiveness to TRH were not significantly different from those with TSH increments greater than 3.9 mU/l in the TRH test.

Two patients had absent TSH (RIA) responses to TRH although basal TSH was detectable by IRMA (Table 6.9). Both were elderly women who died soon after hospital admission.

Table 6.9 Two Patients with Absent TSH Responses to TRH

Patient	1	2
Sex	F	M
Age (Years)	75	67
Diagnosis	Chest infection	Chronic bronchitis
Total T_4 (nmol/l)	102	116
fT_4* (pmol/l)	17.0	22.0
Total T_3 (nmol/l)	0.8	1.2
fT_3† (pmol/l)	2.2	4.3
TSH IRMA (mU/l)	0.5	0.3

Abnormal results underlined. *Amerlex, †Becton Dickinson.

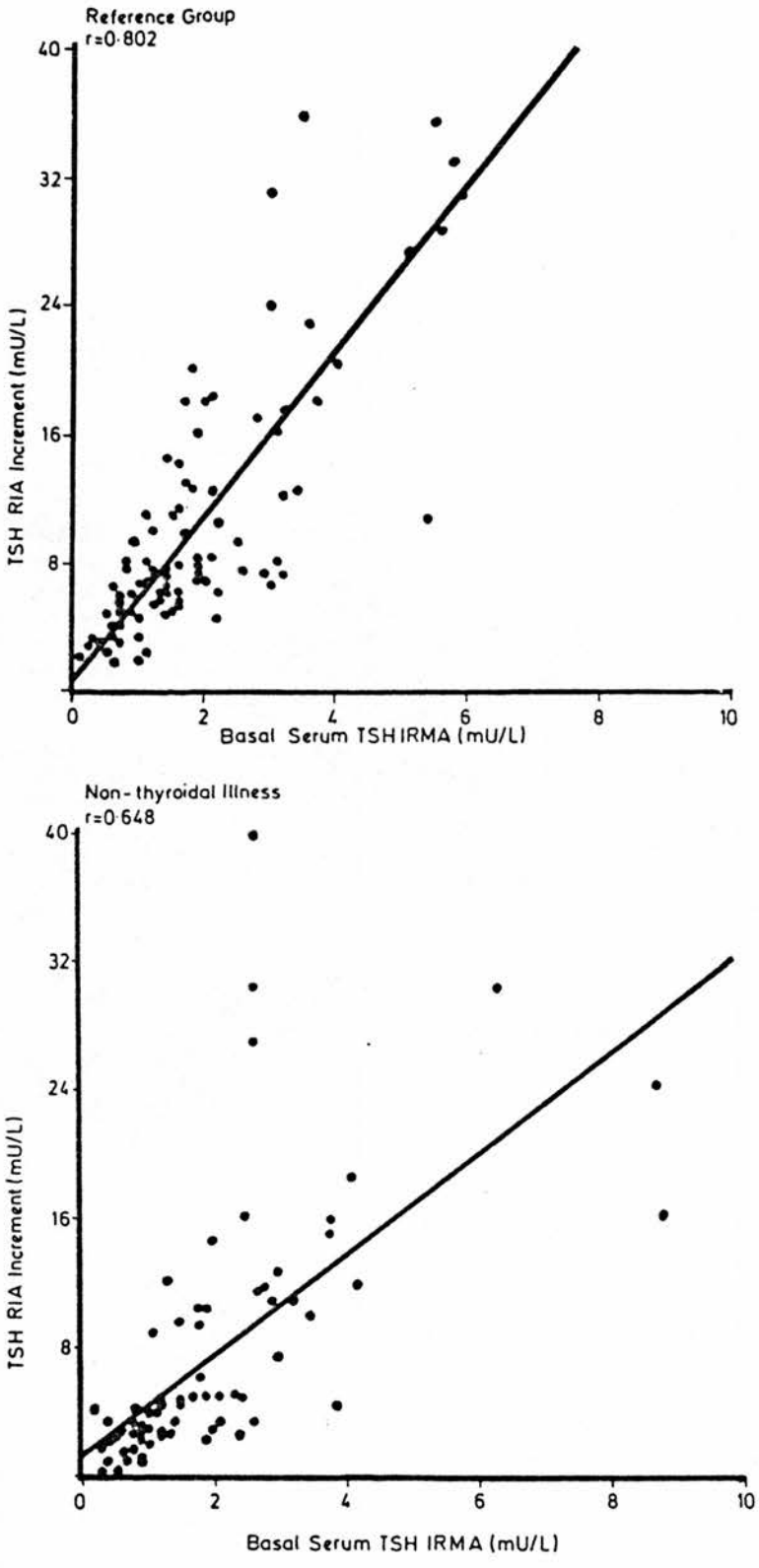


Figure 6.8 The Relationship between Basal TSH (IRMA) and the TSH Increment after TRH in the Reference Group and Patients with Non-thyroidal Illness.

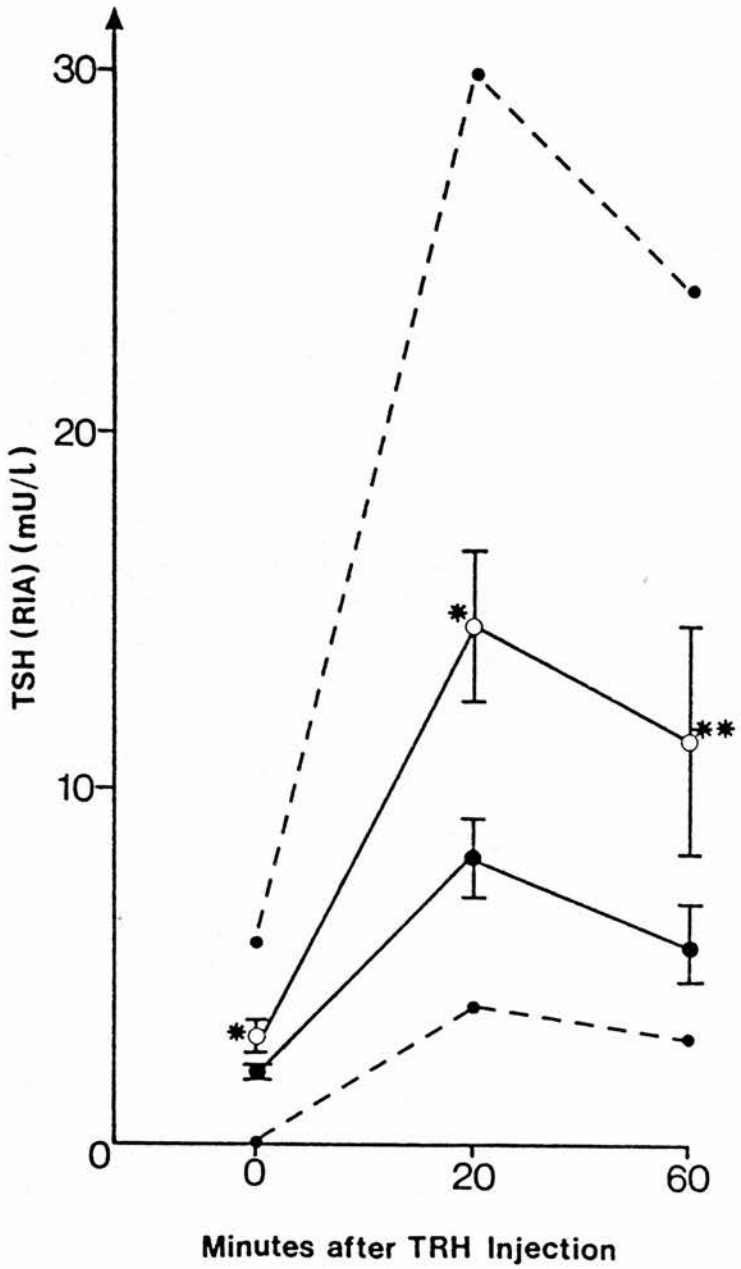


Figure 6.9 The TSH Response to TRH in Male and Female Patients in a Medical Ward.

Significant differences between males (•) and females (o) *p<0.005, **p<0.01. Reference limits are shown by the dashed lines.

6.1.5 Effects of Illness, Sex and Age on Basal TSH IRMA

Concentrations of TSH in the 264 medical ward patients were similar in males and females but slightly lower than the reference group ($p = 0.02$, Mann-Whitney test). This analysis was performed after the exclusion of the 4 patients with overt hypothyroidism and those patients with abnormal TSH values (Tables 6.5 and 6.6). When results for males and females were compared separately with the reference group, it was shown that the decrease in TSH was confined to the male patients ($p<0.002$) and was age-related ($r= -0.265$, $p<0.01$). No age-related decline in basal TSH was found for women in the reference group or from the medical ward.

6.2 SCREENING FOR THYROID DISEASE IN THE ELDERLY

The distinction between features of hypothyroidism and those of old age and/or co-existent systemic illness is difficult clinically, and since primary thyroid disease is more prevalent in the elderly, there is an increased need for a reliable biochemical screening test in this group. Old age and illness may lower total thyroid hormone concentrations in serum (Olsen et al., 1978; Engler et al., 1978; Chopra et al., 1983; Harman et al., 1984) reducing their predictive value. The hyperthyroidism of toxic multinodular goitre is less severe than that of Graves' disease (Toft et al., 1981) and

occurs more commonly in the elderly. Total thyroid hormone concentrations may, therefore, be misleadingly normal in such patients or those with mild hyperthyroidism due to a solitary nodule or to Graves' disease. The TRH test or basal TSH IRMA, as shown in previous chapters, are more sensitive tests of hyperthyroidism since they identify subclinical disease. The role of basal TSH IRMA as a screening test in geriatric patients is investigated in this section.

6.2.1 Patients and Methods

All patients admitted to a geriatric unit (n=63) as in-patients or assessed at a day hospital (n=49) over a seven week period were studied, excluding any patient with known thyroid disease. The mean age of the combined group was 82.4 years (range 65-100) and 69% were women with 31% males. These patients were significantly older than patients from the medical ward, $p<0.001$ (Figure 6.1). Medications and active problems were recorded in all cases.

Blood was obtained from all patients for estimation of TSH (Boots-Celltech IRMA), total T_4 and fT_4 (Amerlex-M) in serum. Patients with abnormal results were re-tested 4-6 weeks later and a TRH stimulation test, total T_3, fT_3 (Amerlex-M) and isotope thyroid scan performed, if appropriate.

6.2.2 Total T_4, Free T_4 and Basal TSH IRMA Results

Abnormal concentrations of total T_4, fT_4 and TSH were found in 9.8%, 4.5% and 8.9% of patients, respectively (Figure 6.10).

(a) Patients with Abnormal Total or Free T_4 but Normal TSH

Nine patients had low total T_4 and/or fT_4 (7 females, 2 males; age range 70-84 years). Four of these patients died within 3 months of the study. Details of their results and clinical problems are given in Table 6.10. One patient (patient 3) was known to have hypopituitarism and had low gonadotrophin levels in serum. Free T_4 concentrations were below the reference range in only 4 of these patients. One patient (patient 9) had a marginally low fT_4 value but normal concentrations of total T_4 and TSH. In 3 patients re-tested 1-2 months later, total and free T_4 concentrations had returned to normal.

One patient (female, 86 years) with normal TSH (1.9 mU/l) had a raised total T_4 (171 nmol/l) but normal fT_4 level (18.1 pmol/l) at first testing. This patient had rheumatoid disease, dementia and confusion and was taking iron tablets and Burinex K. The TBG concentration in this patient's serum was raised (32.5 mg/l) which would account, in part, for the raised total T_4 level.

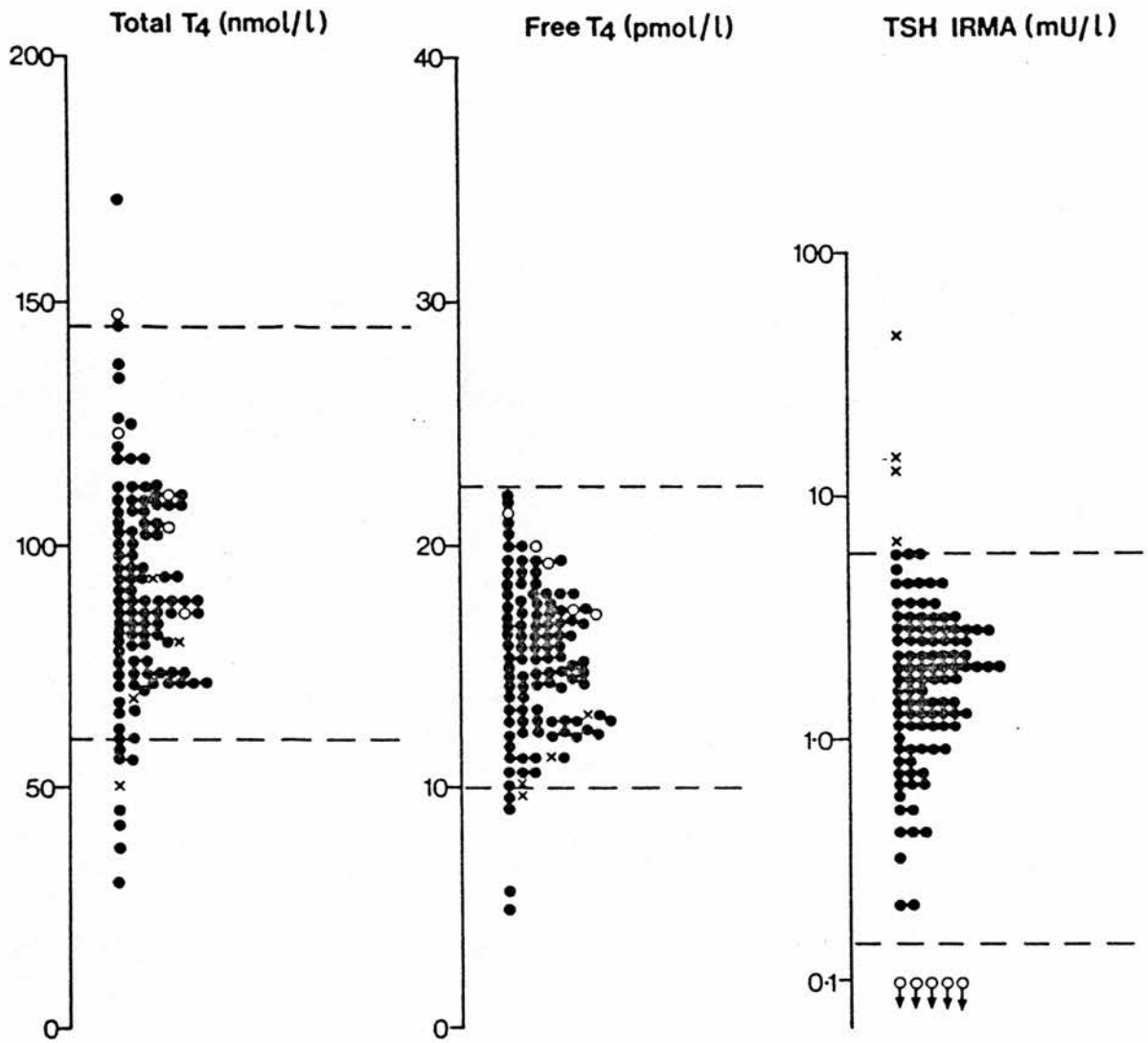


Figure 6.10 Results for Total T_4, Free T_4 (Amerlex-M) and TSH IRMA in 112 Geriatric Patients.

Patients with raised (x) or undetectable (o) concentrations of TSH are as indicated.

Table 6.10 Patients with Low Total or Free T_4 but Normal TSH Concentrations

Patient	Sex	Age	Clinical Problems	Medication	TSH IRMA (mU/l)	T_4 (nmol/l)	fT_4 (pmol/l)
1	F	77	Mild osteoarthritis	Quinine Ponstan	3.7 (4.3	58 91	10.4 11.2)
2	M	84	Recent dietary restriction Confusion	Augmentin	1.2	37 (patient died)	5.6
3	F	75	Hypopituitarism		5.8	30 (now on T_4)	4.8
4	F	73	Atrial fibrillation Cardiac failure Ischaemic heart disease	Frusemide Digoxin Salbutamol Slow K Paracetamol	3.3	42 (patient died)	10.7
5	F	70	Cerebral glioma Left hemiparesis	Moduretic Thioridazine	0.3	56 (patient died)	11.2
6	M	77	Severe Cardiac failure Osteoarthritis	Frusemide Digoxin Spironolactone Allopurinol Distalgesic Terfenadine	3.5	45 (patient died)	9.2
7	F	78	Osteoarthritis Falls	None	0.4	55 (not determined)	13.0
8	F	74	Persistent chest infection Rheumatoid arthritis Ischaemic heart disease	Augmentin Digoxin Diurexan Nitrazepam	2.1 (1.9	59 78	11.5 11.5)
9	F	75	Haematuria Parkinsonism Ischaemic heart disease	Augmentin Chlormethiazole Propranolol Madopar Glycerol Tri-nitrate	4.6 (7.2	73 83	9.9 12.0)

Results consistent with hypothyroidism are underlined.
Results from later testing are shown in brackets.

(b) Patients with Raised TSH Levels

Four patients had raised TSH concentrations with normal serum thyroid hormone levels being present in three (Table 6.11). Patient 4, who had low total T_4 and anti-thyroid antibodies present in his serum, was commenced on thyroxine. Raised TSH concentrations were confirmed 1-2 months later in patients 1-3 and, although total T_4 concentrations remained within reference limits, low fT_4 levels were recorded in two patients.

(c) Patients with Undetectable TSH Levels

Five patients had undetectable TSH values (Table 6.12). None had clinical signs or symptoms of hyper-thyroidism. In one patient total T_4 was raised; this patient died before further investigation for possible hyperthyroidism could be carried out. Normal con-centrations of gonadotrophins were found in her serum making hypopituitarism an unlikely cause of the reduced TSH secretion. In the remaining four patients, tests were repeated 1-2 months later. A TSH (IRMA) response to TRH of greater than 1 mU/l was measured in patient 2, and basal TSH was detectable suggesting some recovery of TSH secretion; the other patients showed no measurable response to TRH. Patient 3 had very low gonadotrophin levels consistent with hypopituitarism but the fT_4 concentration was above reference limits on the second occasion consistent with the presence of hyperthyroidism.

Table 6.11 Patients with Raised TSH Concentrations

Patient	Sex	Age	Clinical Problems	Medication	TSH IRMA (mU/l)	T_4 (nmol/l)	fT_4 (pmol/l)
1	F	78	Diabetes Osteoarthritis Obesity	Cotrimoxazole Navidrex K Metformin	14.2 (10.8	95 107	11.3 11.6)
2	F	82	Depression Dementia Poor mobility	Voltarol retard Navidrex K Lactulose	6.4 (6.5	81 95	12.9 9.7)
3	F	79	Severe Parkinsonism	Madopar Digoxin	44.9 (36.8	68 83	10.0 8.8)
4	M	84	Chronic lymphatic leukaemia Acute episode of chronic bronchitis Dementia	Paracetamol Amoxycillin	12.8 (12.3	50 45	9.7 8.7)

Results consistent with hypothyroidism are underlined.
Results from later testing are shown in brackets.

Table 6.12 Patients with Undetectable TSH in Serum on the First Occasion (Sample 1)

Patient	Sex	Age	Clinical Problems	Medication	Sample	TSH IRMA (mU/l) 0 min	20 min*	T_4 (nmol/l)	fT_4 (pmol/l)	T_3 (nmol/l)	fT_3 (pmol/l)	LH (u/l)	FSH (u/l)
1	F	90	Severe dementia Recent starvation Osteoarthritis	Ibuprofen Lactulose Navidrex K Temazepam	1	<0.12		147	21.3	1.8	4.7	48	>52
										Patient died			
2	F	83	Depression Confusion	Nitrazepam Aspirin	1 2	<0.1 0.2	ND 1.6	111 106	17.2 15.9	1.7 1.5	4.8 3.9	25 23	43 46
3	F	85	Severe dementia Parkinsonism	Digoxin Navidrex K Brufen Lactulose	1 2	<0.1 <0.07	ND 0.14	126 123	20.0 24.7	1.1 1.1	3.1 3.5	< 0.8 < 0.8	1.0 1.4
4	F	88	Myocardial infarction Acute gastrointestinal bleed, Stomach cancer Osteoarthritis Dementia	Paracetamol Timoptol Adrenaline Imipramine Metaclopramide	1 2	<0.1 <0.07	ND <0.07	87 87	19.4 16.4	1.6 1.0	4.8 3.5	21 8	46 40
5	F	91	Severe dementia Immobility and falls	Voltarol Retard Folic acid Vitamin B_{12}	1 2	<0.1 <0.07	ND <0.07	104 110	17.4 20.1	2.0 2.0	12.2 12.9	21 14	42 40

Post-menopausal Reference Ranges for LH and FSH are 30-115 u/l
Results consistent with hyperthyroidism are underlined
*After TRH, ND = not determined.

This patient also had severe dementia, Parkinsonism and depression, and she died subsequently. The two remaining patients had normal isotope thyroid scans and neither had detectable thyroid autoantibodies in serum. Patient 4 was seriously ill and maintained on a complex drug regime; these factors may have contributed to her low TSH secretion. Patient 5 was comparatively healthy but had a persistently elevated fT_3 concentration in serum.

Comparison of TSH (IRMA) values in the geriatric group with the reference group showed no significant difference (Mann-Whitney test, $p=0.17$): patients with undetectable TSH or values greater than 10 mU/l were excluded prior to this analysis.

6.2.3 Summary and Discussion

Relatively few geriatric patients (3.5%) had raised TSH concentrations, as found in the medical ward (6.0%). This proportion was more consistent with the known prevalence of primary hypothyroidism than that suggested by the number of low results for thyroid hormone measurements. However, the possibility that patients with primary thyroid failure were missed using TSH due to drug- or illness-related suppression of TSH secretion has to be considered. None of the patients in the two studies who

had low total T_4 but normal TSH were taking glucocorticoids or dopamine agonists, and only one patient was taking phenytoin which may also suppress TSH secretion (Franklyn et al., 1985). This reduces the possibility that overt hypothyroidism was missed by the TSH measurement. Fasting may suppress TSH secretion into the reference range in patients with mild hypothyroidism (Borst et al 1983); and this cannot be excluded in the patients studied. However, the basal TSH levels in those patients with low total or free T_4 but normal TSH were not significantly higher than the reference group. The low thyroid hormone levels could equally be due to the low serum protein and albumin concentrations present and the drug therapy used in these patients. Low total T_4 concentrations were also related to higher mortality in the geriatric patients as reported by Slag et al. (1981a). Measurement of TSH provides a more specific test of hypothyroidism than measurement of thyroid hormones in elderly patients and those with co-existent illness.

Thyrotrophin concentrations reportedly increase during the recovery phase of systemic illness (Bacci et al., 1982) but this cannot account for the raised TSH values in admission samples from patients in the medical ward nor the persistently raised TSH concentrations demonstrated in the study of geriatric patients. Metaclopramide , a dopamine receptor blocking agent and

anti-emetic, is reported to stimulate TSH secretion (Scanlon et al., 1981) but none of the patients described with raised TSH were taking this drug.

A larger proportion of geriatric patients than medical in-patients had undetectable TSH concentrations. Although an age-related decrease in basal TSH and the response to TRH was demonstrated in men, undetectable levels occurred most frequently in elderly women; thyroid hormone results were consistent with the presence of sub-clinical hyperthyroidism in half of these patients. In others, severe depression, dementia and prolonged fasting may have contributed to the suppression of TSH secretion. None of these patients were taking dopamine, glucocorticoids or phenytoin.

6.3 THYROID FUNCTION TESTS IN ELDERLY PATIENTS WITH CHRONIC OBSTRUCTIVE AIRWAYS DISEASE

Approximately 20% of admissions to the medical ward (Section 6.1) were patients with respiratory dysfunction, a proportion of whom had low serum concentrations of total and free T_4 by analogue assay (Tables 6.2 and 6.3). It has been suggested that chronic hypoxia in patients with chronic obstructive airways disease (COAD) can produce abnormalities of hypothalamic-pituitary function (Semple et al., 1979, 1981, 1983), the main effects being on the hypothalamic-pituitary testicular axis. However, in contrast to reports of reduction (Snyder &

Utiger, 1972b) and even failure (Davies et al., 1985) of TSH responses to TRH in the elderly with severe illness, delayed TSH responses to TRH (i.e. 60 min TSH level greater than that 20 min after TRH) have been reported in hypoxic patients with COAD or pulmonary fibrosis (Semple et al., 1981 & 1984). The contribution of hypoxia to alterations in thyroid function tests and pituitary hormone secretion has been investigated in this section by studying patients with severe COAD and age-matched control patients with normal respiratory function.

6.3.1 Patients and Methods

Patients: The group with COAD comprised 20 patients (16 males, 4 females; mean age 68 years, range 42-81) admitted to hospital with acute exacerbations of their disease. The forced expiratory volume in one second (FEV_1) and the forced vital capacity (FVC) expressed as percentages of predicted normal values were less than 40% and 65%, respectively in all 20 patients, indicating severe respiratory impairment. The control group comprised 15 ambulatory patients (10 males, 5 females; mean age 73 years, range 57-83) about to be discharged from hospital. These patients were convalescing from cardiovascular problems (angina, syncope, cardiomyopathy, deep vein thrombosis, small myocardial infarction), minor cerebrovascular accidents which had occurred 6 months previously, or dementia. All 15 had FEV_1 and FVC measurements within

20% of predicted normal values and had no sign of respiratory disease. None of the 35 patients had clinical evidence of thyroid disease and none was receiving oral corticosteroids or other drugs known to affect the hormone measurements.

Methods: Weight and ideal weight were measured in all those studied and blood gas analysis was performed on arterial samples from the patients with COAD while breathing room air. Reference ranges were those used routinely and details of these are shown in Table 6.13.

Table 6.13 Details of Patients Studied

	Mean (Range)	
	COAD Patients (n = 20)	Control (n = 15)
Age (yr)	68 (42-81)	73 (57-83)
Weight (Kg)	55 (44-70)	68 (32-102)
% Ideal Weight	81 (67-110)	95 (53-122)
PaO_2 (kPa) (Ref. range 12.0-15.0)	7.1 (4.3-12.6)	ND
$PaCO_2$ (kPa) (Ref. range 4.5-6.1)	7.3 (4.2-10.5)	ND
$[H^+]$ (nmol/l) (Ref. range 34-44)	42 (32-60)	ND

ND = Not determined

Blood samples were taken 2-3 days after hospital admission in those with COAD. Venous blood was taken in the afternoon at 0 min and then at 20 and 60 min after TRH when patients were relaxed and resting.

Basal measurements of total T_4 and T_3 were performed by in-house RIA and TSH by the Boots-Celltech IRMA. Free thyroid hormones were measured by the Amerlex analogue methods. The TSH response to TRH was measured by in-house RIA. Pituitary hormones (prolactin, growth hormone (GH), LH, FSH) and testosterone were measured in serum by staff at the Immunoassay Section of this department using in-house RIA methods (Beckett et al., 1983a; Corker et al., 1978). Between-assay coefficients of variation for these methods ranged from 4-12%. Reference ranges for prolactin and GH were derived from middle-aged males and females attending an outpatient endocrine clinic; those for LH, FSH and testosterone in males were derived from middle-aged healthy men attending for sterilisation or vasectomy.

6.3.2 Thyroid Hormone and Basal TSH Results

Results for total and free thyroid hormones and TSH (IRMA) are shown in Figure 6.11. None of these measurements differed significantly (Mann-Whitney test) between COAD and control patients. Several low results for total and free T_3 were found in both patient groups. However, only patients with COAD had total T_3 and fT_3 values below 0.8 nmol/l and 3.0 pmol/l, respectively. Two patients had marginally elevated basal TSH levels: one with COAD (8.2 mU/l) and one control (8.9 mU/l); total and free thyroid hormone concentrations were within normal limits in both patients.

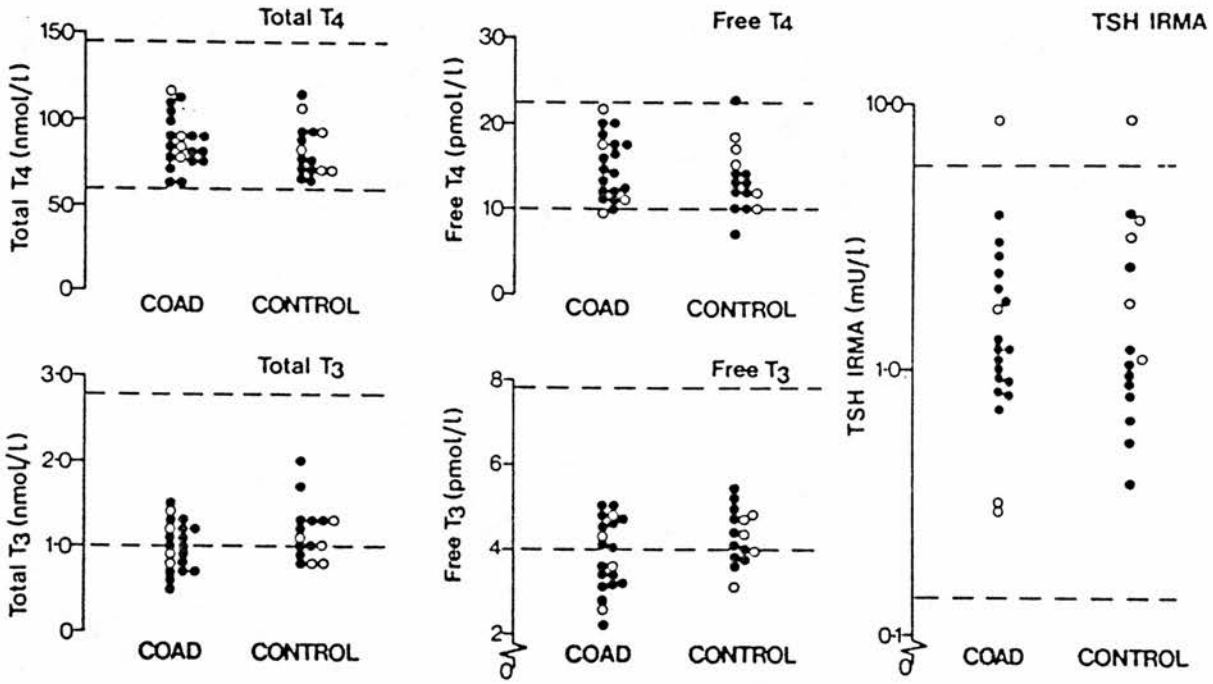


Figure 6.11 Thyroid Hormone and Basal TSH (IRMA) Concentrations in Patients with Chronic Obstructive Airways Disease (COAD) and Controls.

Results are shown for male (•) and female (o) patients; free thyroid hormones were measured by Amerlex kits.

6.3.3 TSH Responses to TRH

Results for TSH (RIA) pre- and post-TRH in control patients and those with COAD are shown in Figure 6.12. In general, higher TSH increments 20 min after TRH were found in females than in males ($p<0.05$). There was no significant difference in the TSH increment after TRH between patients with COAD and controls, even excluding the female patients (Table 6.14).

Three patients with COAD (2 males, 1 female) had absent TSH responses to TRH (20 min increment less than 1 mU/l). The female patient died suddenly with acute respiratory failure 3 days after the study. Eight other patients with COAD (7 males, 1 female) had reduced responses (less than 3.9 mU/l increment). In the control group, reduced responses were found in 8 male patients. A delayed TSH response to TRH (60 min level greater than the 20 min) was found in 2 patients with COAD and 5 controls.

6.3.4 The Secretion of Pituitary Hormones and Testosterone

In normal individuals there is an increase in serum prolactin (Cowden et al., 1979) but little secretion of GH in response to TRH (Gomez-Pan et al., 1979). However, systemic illness may alter the release of pituitary hormones in response to TRH as shown in patients with chronic renal failure (Gomez-Pan et al., 1979; Beckett et al., 1983a).

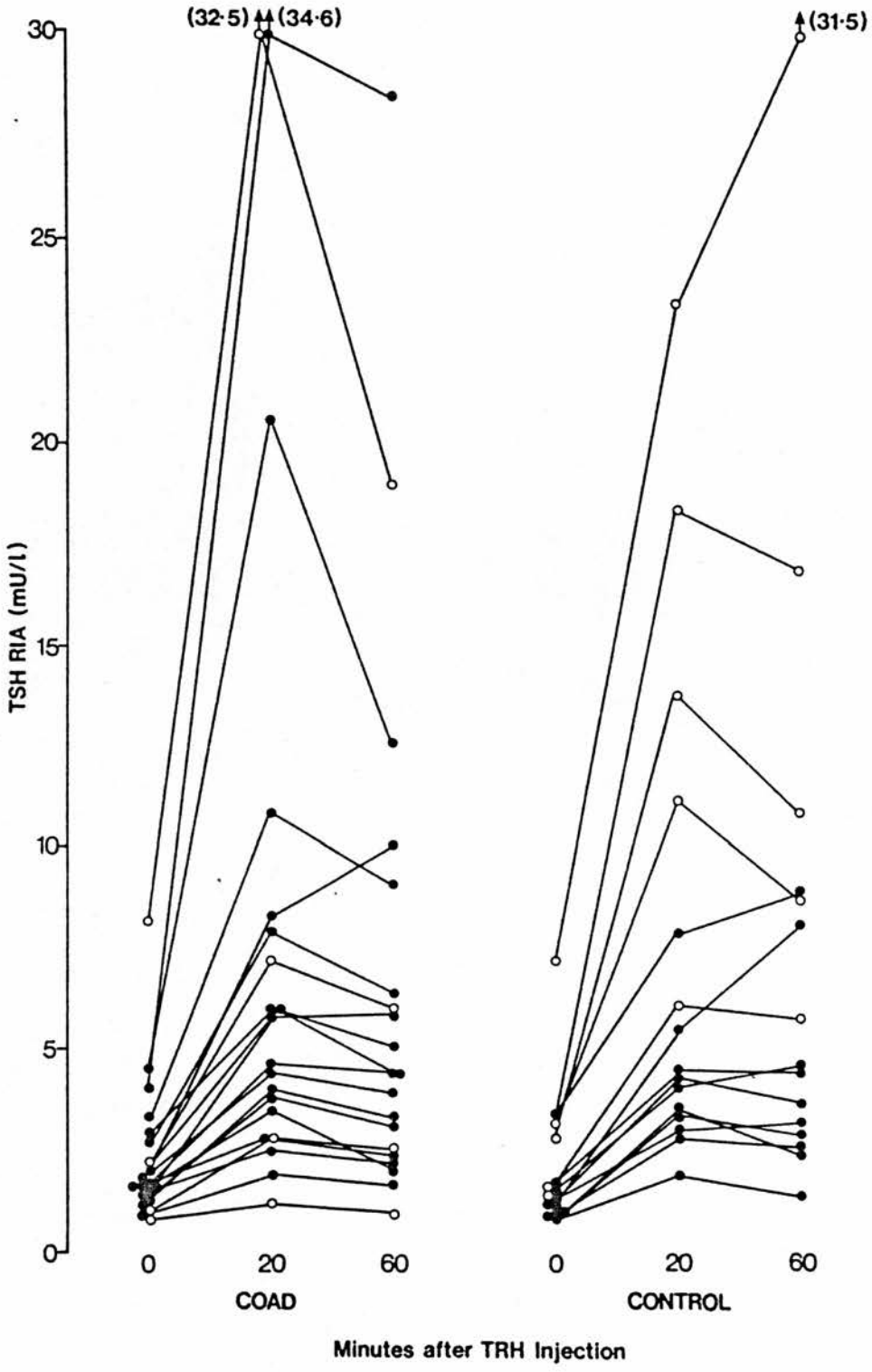


Figure 6.12 The TSH Response to TRH in Patients with COAD and Controls.

(Males (●), females (o)).

Table 6.14 Basal Serum Hormone Concentrations and Increments 20 min after TRH

Test	Reference Ranges	Mean (Range) COAD Patients	Mean (Range) Controls
Basal TSH RIA	<5.7 mU/l	2.3 (<0.9- 8.2)	2.0 (<0.9- 7.2)
Basal TSH IRMA	0.14 - 5.9 mU/l	1.8 (0.3- 9.8)	2.1 (0.5- 8.9)
TSH increment	3.9 - 30 mU/l	6.2 (1.0-30.6)	5.6 (1.4-16.3)
[Males only		5.8 (1.0-30.6)	2.8 (1.4- 4.6)]
Basal Prolactin	60- 390 mU/l	212 (<32-961)	302 (73 - 856)
PRL increment	females: >233 mU/l*	589 (103-2584)	634 (183 -1739)
[Males only	males: > 38	487 (103-1191)	476 (183 - 943)]
Basal GH	often <1.0 mU/l but variable	2.1 (<0.6-11.2)	1.5 (<0.7- 7.9)
GH increment	no response	2.1 (0 - 9.5)	5.9 (0 - 45)
[Males only		1.9 (0 - 9.5)	1.2 (0 - 4.7)]
Testosterone (males)	10 - 30 nmol/l	10.7 (3.0 -19.5)	11.0 (1.8-21.9)
LH (males)	1.5 - 9.0 U/l	9.8 (2.3 -14.9)	9.1 (2.1-34.1)
[LH (males) excl.**		9.8 (2.3 -14.9)	6.3 (2.1- 9.1)]
FSH (males)	1.5 - 9.0 U/l	8.8 (2.7 -30.5)	5.4 (1.8- 7.6)

*From Cowden et al., 1979.
**A significant difference ($p<0.02$) was found for LH after exclusion of one control patient, see text. No significant differences were found for the other tests (Mann-Whitney test).

In this study, there were no significant differences between basal concentrations of prolactin and GH nor their increments after TRH, comparing COAD and control patients (Table 6.14). Many patients showed a post-TRH rise in prolactin of greater than 700 mU/l (4 COAD, 6 controls) or a rise in GH of greater than 1 mU/l (10 COAD, 7 controls). The peak responses for prolactin and GH were greater in females than males ($p<0.05$) but exclusion of the females from the data analysis still showed no difference between the COAD and control groups.

Concentrations of basal LH, FSH and testosterone in male patients did not differ significantly between the groups (Table 6.14). One control patient had the lowest testosterone concentration (1.8 nmol/l) and the highest LH concentration (34.1 U/l) in the study, indicating primary gonadal failure. Exclusion of this patient from the statistical analysis revealed significantly higher LH concentrations in COAD men compared to male controls although testosterone concentrations remained similar (Table 6.14). Low concentrations of testosterone were found in 7 male patients with COAD and 5 control patients.

6.3.5 Correlations between Hormone Measurements, Indices of Respiratory Function and Body Weight

None of the serum hormone measurements made in the group with COAD correlated significantly with arterial blood gas tensions (Pa_{O_2} and Pa_{CO_2}), % FEV_1 or % FVC.

Patients with high arterial hydrogen ion concentrations tended to have high basal GH levels but more patients would need to be studied to improve the distribution of data points and allow firm conclusions to be drawn (Figure 6.13). Significant correlations were observed for the TSH (RIA) increment after TRH and the basal TSH measured by IRMA when compared with the percentage ideal body weight in patients with COAD (Figure 6.14). In the correlation of TSH increment with percentage ideal weight (Figure 6.14A), a logarithmic transformation of the data was performed to normalise the data distribution; this reduced the correlation coefficient from $r=0.675$ in a linear plot to $r=0.516$, as shown. One patient (percentage ideal weight 110, TSH IRMA 8.8 mU/l) was excluded from Figure 6.14B as an outlier, reducing the correlation coefficient from $r=0.724$ originally, to $r=0.465$, as shown. For both correlations, statistical significance ($p<0.05$) was maintained.

In summary, both delayed and reduced TSH responses to TRH were found in patients with COAD and controls. Such changes were therefore not specific to hypoxic patients. The fact that absent TSH responses were found only in the patients with COAD and not controls may be explained by the additional suppressive effect of malnourishment as suggested by the correlation of TSH response with percentage ideal weight. The changes related to hypoxia reported by others may represent an

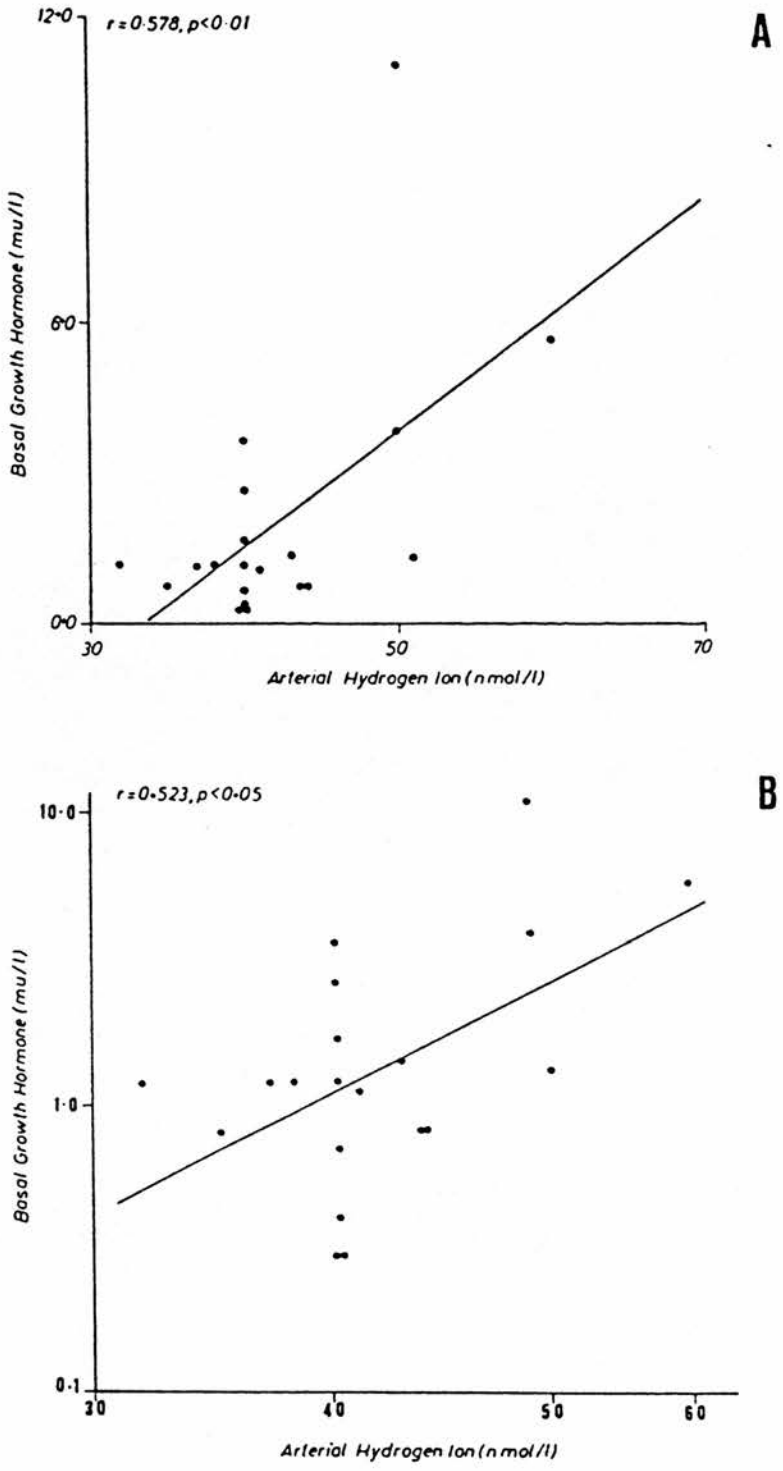


Figure 6.13 Correlation between Growth Hormone Concentrations and the Arterial Hydrogen Ion Concentration in Patients with COAD.

A: Linear regression; B: Correlation after logarithmic transformation of both variables.

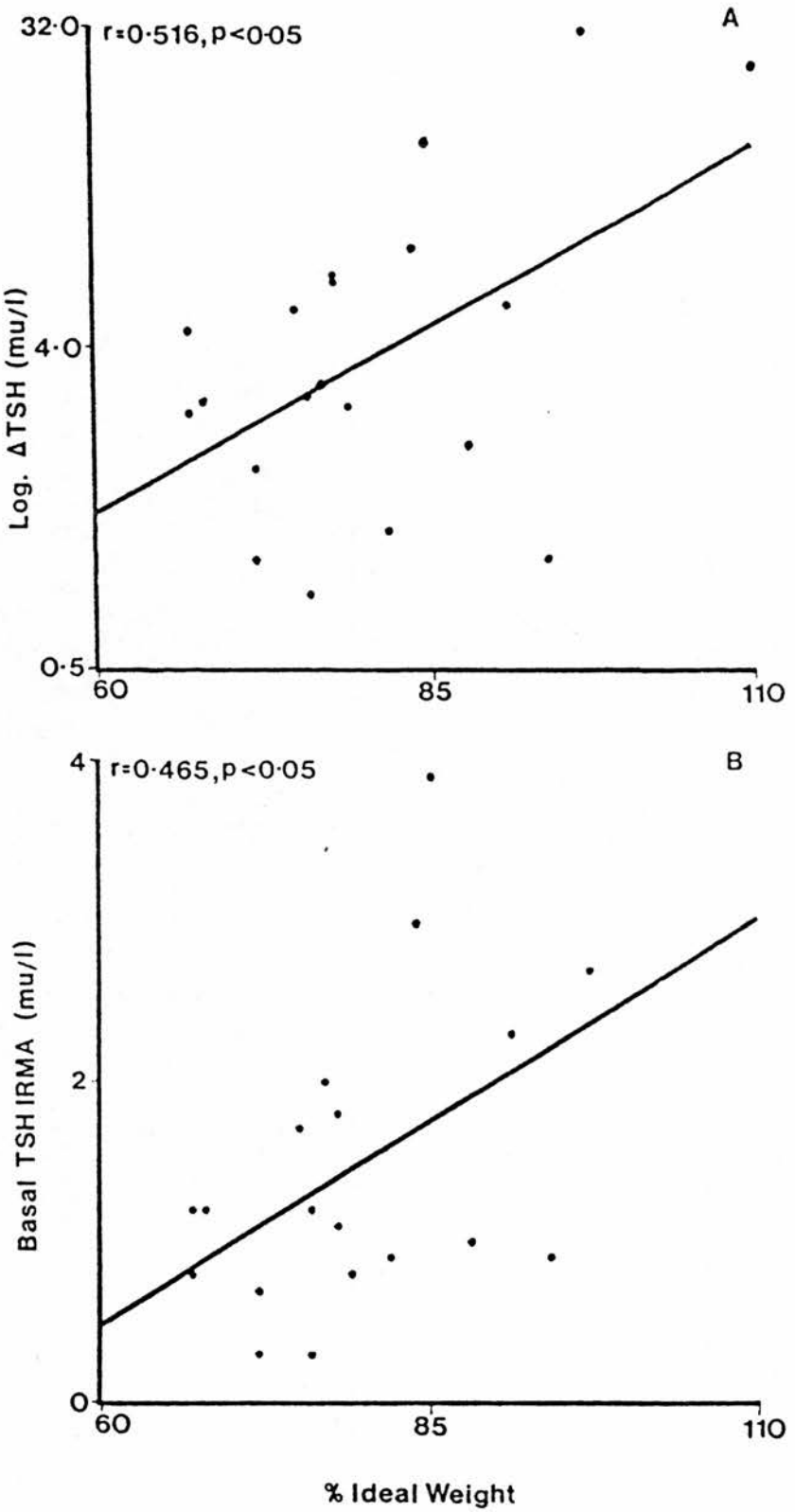


Figure 6.14 Correlations between (A) the Logarithm of the TSH (RIA) Increment after TRH and (B) Basal TSH IRMA with the % Ideal Weight of Patients with COAD.

early onset of age-dependent changes in COAD since the male patients in this study were significantly older and more underweight ($p<0.01$) than those studied previously by Semple et al. (1979, 1981, 1983).

6.4 THYROID STATUS IN CHRONIC RENAL FAILURE

In patients with chronic renal failure (CRF), low concentrations of total thyroid hormones have been reported despite normal TBG concentrations (Lim et al., 1977; Gomez-Pan et al., 1979; Beckett et al., 1983a). Measurements of fT_4 using the FT4I or analogue RIA kits have yielded low results (Lim et al., 1977; Melmed et al., 1982; Beckett et al., 1983a) while fT_4 measured by indirect dialysis procedures have either been low (Melmed et al., 1982) or not significantly different from controls (Beckett et al., 1983a). Using direct dialysis methods, values have been reported as normal (Lewis, 1979) or high (Liewendahl et al., 1984). The presence in serum of weak inhibitors of protein-binding may account for the disparity of dialysis fT_4 results found in CRF since different dilutions of serum have been employed by different investigators. There is, as yet, no consensus on the true levels of circulating free thyroid hormones in patients with CRF.

The HPT axis appears to be abnormal in CRF and the production of TSH in response to TRH is frequently diminished. In contrast, GH and prolactin production

is enhanced (Gomez-Pan et al., 1979; Beckett et al., 1983a). The clinical utility of sensitive TSH measurement by IRMA remains to be investigated in this group of patients.

6.4.1 Patients Studied

Thirty-six patients (15 males, 21 females) with CRF who were receiving treatment by intermittent haemodialysis were studied. The age range was 14-70 years (mean 45). Serum samples were collected prior to their haemodialysis treatment to avoid artefacts in the measurement of thyroid hormones due to heparin-anticoagulation of the patients. The results for the routine clinical chemistry analyses are given in Table 6.15 demonstrating the reduced excretion of urea, creatinine, uric acid, potassium, phosphate, and the metabolic acidosis in these patients. Some patients also had results suggestive of hyperparathyroidism secondary to renal disease (i.e. low serum calcium, raised serum alkaline phosphatase). Serum protein concentrations were well-maintained in this group of patients, values below the reference limits for total protein and albumin occurring in only 4 and 2 patients, respectively.

Table 6.15 The Clinical Chemistry of Patients with CRF

Analyte	Reference Range	CRF Patients (Mean ± SD)
Urea	2.5 - 6.6 mmol/l	30.4 (8.7)
Creatinine	55 - 150 µmol/l	1205.5 (242)
Uric acid (males)	0.12-0.42 mmol/l	0.55(0.12)
(females)	0.12-0.36 mmol/l	
Na	132 - 144 mmol/l	137.8 (3.8)
K	3.3 - 4.7 mmol/l	5.5 (1.0)
Total CO_2	24 - 30 mmol/l	22.5 (4.3)
Calcium	2.12-2.62 mmol/l	2.38(0.32)
Phosphate	0.8 -1.4 mmol/l	1.93(0.63)
Total protein	60 - 80 g/l	65.8 (5.3)
Albumin	36 - 47 g/l	40.2 (4.6)
Bilirubin	2 - 17 µmol/l	5.1 (1.3)
ALT	10 - 40 U/l	20.2 (14.4)
Alkaline phosphatase	40 - 100 U/l	142.5 (158.2)
GGT (males)	10 - 55 U/l	23.9 (26.9)
(females)	5 - 35 U/l	

6.4.2 Results for Thyroid Function Tests

The number of patients with abnormal concentrations of thyroid hormones and basal TSH by Boots-Celltech IRMA are shown in Table 6.16. Compared with general medical in-patients (Table 6.1), four to five times as many patients with CRF had low concentrations of total T_4 or fT_4 whereas the proportion of patients with

Table 6.16 The Number of Abnormal Thyroid Function Tests in Patients with CRF

Test	Result	
	Low	High
Total T_4	15(42%)	0
fT_4 (Amerlex)	24(67%)	0
Total T_3	10(28%)	0
fT_3 (Becton Dickinson)	25(69%)	0
TSH IRMA	1*(3%)	1(3%)

*Undetectable

low total or free T_3 concentrations were similar. Results for serum total T_4, fT_4 (Amerlex) and TSH are shown in Figure 6.15. One female patient aged 50 years had a marginally raised basal TSH concentration (7.7 mU/l). The patient with an undetectable TSH by IRMA also had an absent response to TRH injection. She had been treated with radioiodine in the past for Graves' disease. Basal TSH concentrations in patients with CRF were not significantly different from the reference group and no sex-difference was found.

6.4.3 Measurement of Free Thyroid Hormones

Results for fT_4 and fT_3 in serum by different analogue RIA methods and equilibrium dialysis are shown in Figure 6.16. Most values for fT_4 by dialysis using serum diluted 1:20 were within reference limits, and the mean fT_4 value (10.6±2.9 pmol/l) in patients with CRF was not significantly different from that of 40 euthyroid out-patients (11.7±2.8 pmol/l) whose sera were dialysed in the same assays. Using the analogue fT_4 kits, the majority of patients had low results. The Becton Dickinson and Corning Magic kits produced low fT_4 results in more than 80% of patients.

Dialysis fT_3 measurements were performed using sera diluted 1:2 with buffer and low results were found in 47% of patients (Figure 6.16). The fT_3 values in this group were significantly lower (Mann-Whitney test,

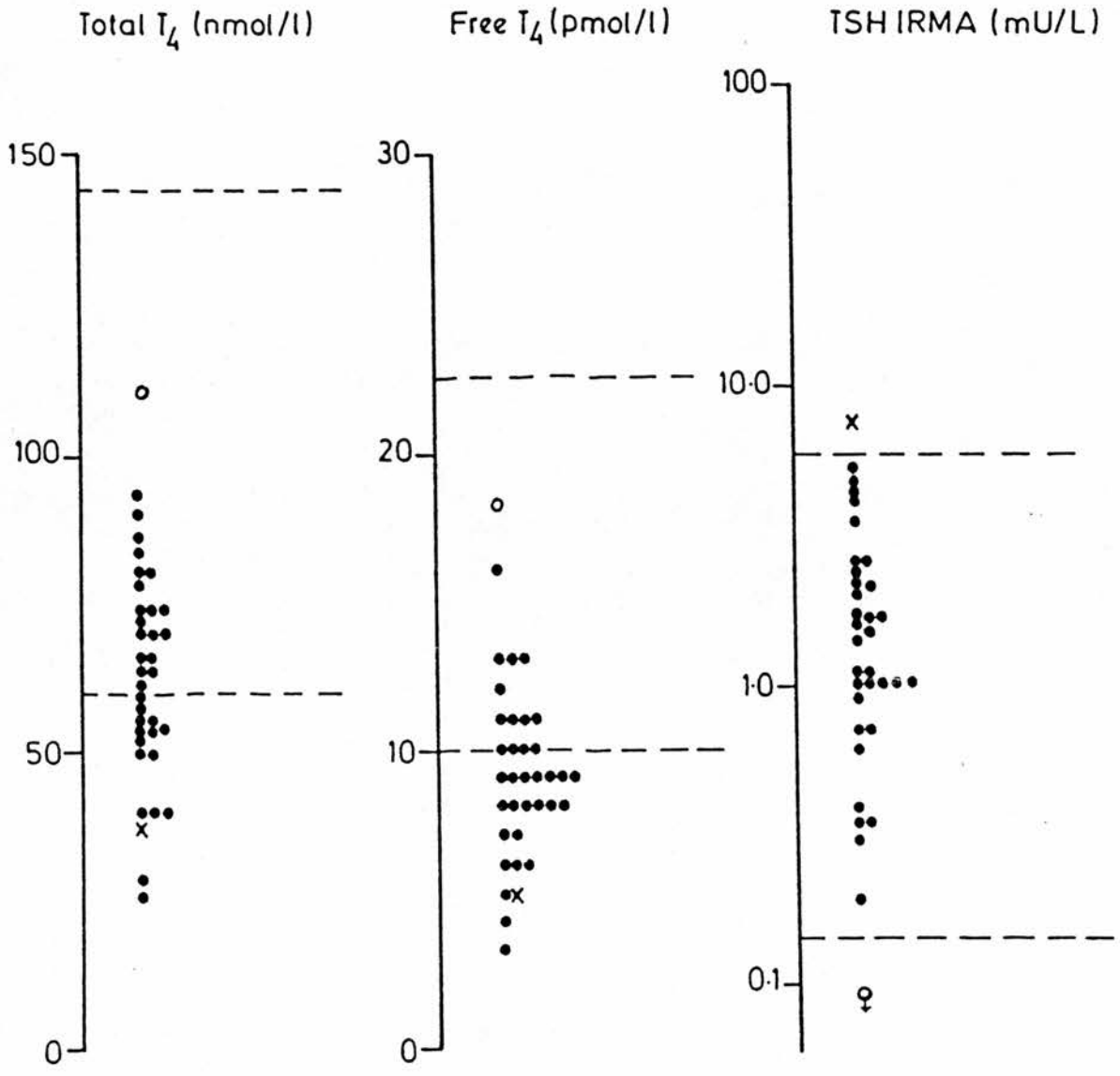


Figure 6.15 Total T_4, Amerlex Free T_4 and Basal TSH (IRMA) Concentrations in 36 Patients with Chronic Renal Failure.

One patient (o) had a past history of thyroid disease and one had a raised TSH value (x).

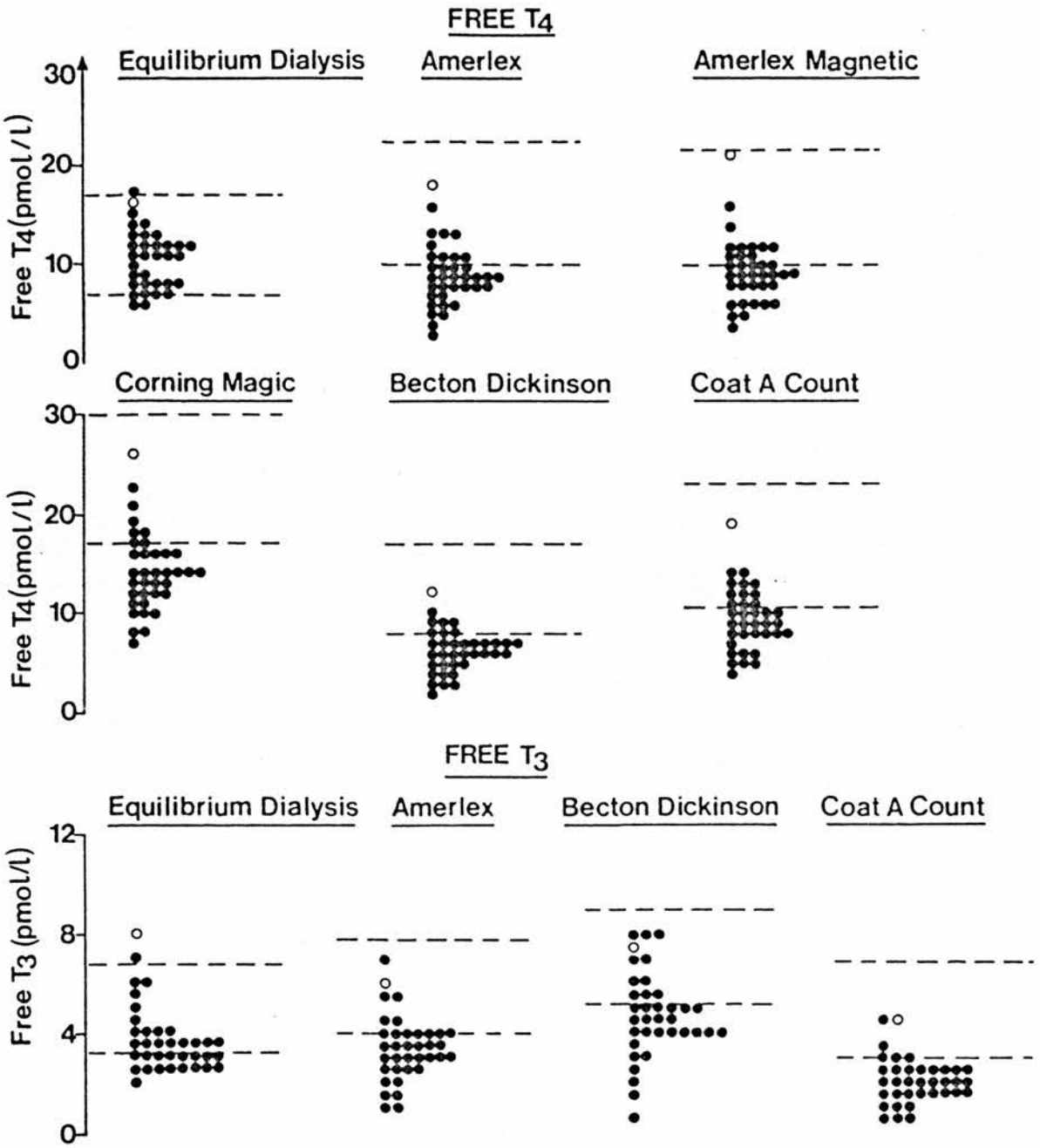


Figure 6.16 Free Thyroid Hormone Concentrations by Dialysis and Analogue RIA Methods in Patients with Chronic Renal Failure.

One patient (o) had an undetectable basal TSH IRMA (see text).

$p<0.005$) than values for 18 samples from euthyroid out-patients dialysed in the same assays. Low fT_3 results were found in more patients by analogue assay than by equilibrium dialysis. The analogue method producing most results within reference limits was the Becton Dickinson kit. This may reflect the greater albumin-dependence of fT_3 results by this method; the majority of these patients had normal concentrations of serum albumin.

The possible effect of endogenous inhibitors of thyroid hormone binding in CRF was evaluated by studying the effect of serum dilution on fT_4 and fT_3 values measured by dialysis-RIA. For sera containing a weakly bound inhibitor, diluting the serum (and the inhibitor) should allow more thyroid hormone to bind to serum protein with a concomitant decrease in fT_4 and fT_3 as demonstrated previously for NEFA (Figure 6.7) and various drugs e.g. salicylate, furosemide (Nelson & Weiss, 1985; Stockigt *et al.*, 1985). Dilution profiles for 8 sera from patients with CRF (NEFA concentrations, 0.56±0.4 mmol/l) showed a decrease in fT_4 with increasing serum dilution (Figure 6.17). However, unlike the situation with raised NEFA levels in serum, high fT_4 concentrations in undiluted serum occurred in only one of the eight patients with CRF (NEFA = 0.30 mmol/l). Comparison of fT_4 values using neat serum (13.3±4.6 pmol/l) and a 1:20 dilution of serum (7.3±2.1 pmol/l) suggested a 50% decrease ($p<0.01$) whereas fT_3 values did not change significantly for dilutions up

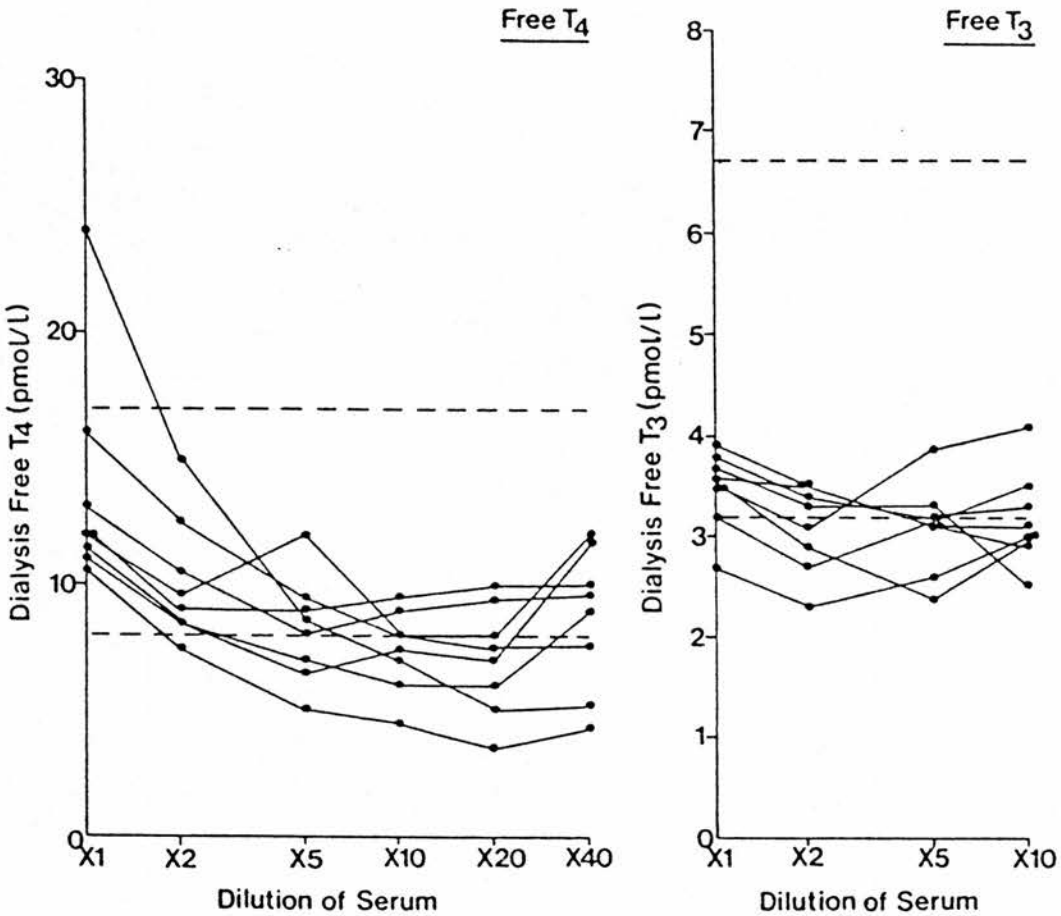


Figure 6.17 The Effect of Serum Dilution on Dialysis Free T_4 and T_3 Values in Patients with Chronic Renal Failure.

to ten-fold (Figure 6.17). True fT_4 values in CRF are therefore unlikely to be lower than in healthy individuals. Other workers have also reported slightly high fT_4 and low fT_3 values by equilibrium dialysis in patients with CRF (Liewendahl et al., 1984; Wang et al., 1985).

6.4.4 Correlations with Plasma Urea Concentration

The relationship between thyroid function test results and the plasma urea concentration used as an index of the severity of renal failure was examined (Table 6.17). The patient with a past history of Graves' disease was excluded from this analysis. Correlations with plasma creatinine concentrations were less significant.

Table 6.17 Correlations between Thyroid Function Test Results and the Plasma Urea Concentration

Correlate with plasma Urea Concentration	Correlation Coefficient (r)	Significance
Total T_4	-0.545	$p<0.01$
Amerlex fT_4	-0.341	$p<0.05$
Dialysis fT_4	-0.280	NS
Dialysis fT_3	-0.374	$p<0.05$
Amerlex fT_3	-0.228	NS
Total T_3	-0.167	NS
TSH IRMA	-0.051	NS

6.4.5 Summary and Discussion

As demonstrated in these younger patients with renal failure, fT_3 concentrations (dialysis) as well as total T_3 concentrations decline in non-thyroidal illness. This has been explained by increased peripheral conversion

of T_4 to rT_3 (Chopra, 1975), and is thought to be a general finding in sick euthyroid patients. However, it probably depends on the severity of disease since Boye & Weeke (1986) have found normal fT_3 levels by ultra-filtration-RIA of undiluted serum taken from medical admissions. Many of the low total T_4 results seen in patients from the medical ward may be due to low concentrations of binding proteins. However, in patients with CRF over 40% of patients had low total T_4 but most had normal concentrations of total protein and albumin in serum. In contrast, patients with exacerbations of COAD and chronic hypoxia maintained normal total T_4 concentrations compared to age-matched controls, highlighting the different effects on thyroid hormone levels in these two chronic disease states. In both chronic illnesses however, basal TSH measurement indicated euthyroidism. This was consistent with the normal fT_4 levels measured by dialysis in patients with CRF. Free thyroid hormone measurements by analogue RIA give misleadingly low results in such patients.

In the specific disease groups studied and in the patients from the general medical ward and the geriatric hospital, basal serum.TSH concentrations measured by IRMA were not significantly different from those of euthyroid out-patients.

Chapter 7

THE BIOCHEMICAL MONITORING OF THYROID STATUS IN PATIENTS TREATED FOR THYROID DISEASE

In this chapter, the biochemical monitoring of patients treated for primary thyroid disease is investigated with particular reference to the role of basal TSH measurements by IRMA.

7.1 THYROID HORMONES AND THYROTROPH RESPONSIVENESS AFTER TREATMENT FOR THYROTOXICOSIS

In the early months following treatment of thyrotoxicosis, the return of thyrotroph responsiveness to exogenous TRH lags behind the fall in thyroid hormone levels (Ballabarba et al., 1972) due to atrophy of the thyrotrophs (Bakke et al., 1964). Measurements of TSH may not, therefore, identify the onset of hypothyroidism and the need for T4 replacement after radioiodine or thyroid surgery (Toft et al., 1974 & 1978). The high sensitivity and specificity of immunometric methods for measuring TSH allows more detailed assessment of thyrotroph function. This was illustrated in Chapter 4 by the demonstration of partial thyrotroph responsiveness to TRH in a proportion of patients with subclinical hyperthyroidism who had undetectable basal TSH concentrations by IRMA. This incomplete thyrotroph suppression was not detected using TSH RIA. In the following section, the time-course of thyrotroph recovery after treatment of thyrotoxicosis is studied with measurements of TSH after TRH injection by RIA and IRMA.

7.1.1 Patients and Methods

Twelve patients (11 female, 1 male; 11 with Graves' disease and 1 with multinodular goitre) were investigated before treatment with ^{131}I and thereafter at four week intervals for 12 weeks. In addition, one woman with Graves' disease was studied during treatment with carbimazole. At each attendance a clinical assessment and TRH test were performed. Thyrotrophin was measured by in-house RIA and the Boots-Celltech IRMA. Total and free thyroid hormone concentrations (Amerlex) were measured in the basal sample.

7.1.2 Thyroid Function and TRH Test Results

As shown in Table 7.1, after treatment for thyrotoxicosis, 8 patients became symptomatically and biochemically hypothyroid, 4 were clinically euthyroid with normal thyroid hormone levels but absent TSH responses to TRH at 8 and 12 weeks post-therapy, and one patient remained hyperthyroid.

The onset of hypothyroidism, as judged by low total and free thyroid hormone levels, was noted at 4, 8 and 12 weeks post-therapy in 1, 5 and 2 patients, respectively (Table 7.1; patients 1-8), but high TSH levels occurred in only 5 of these 8 patients. In the remaining 3 patients, TSH concentrations were either normal or low and responses to TRH were absent in two (patients 4 and 6) and normal in one (patient 5).

Table 7.1 Thyroid Hormone and TRH Test Results Before and After ^{131}I Therapy for Thyrotoxicosis

Patient No	^{131}I Dose (MBq)	*Weeks after ^{131}I	Total T_4 (nmol/l)	Total T_3 (nmol/l)	fT_4 (pmol/l)	fT_3 (pmol/l)	TSH RIA (mU/l) 0 min	TSH RIA (mU/l) 20 min	TSH IRMA (mU/l) 0 min	TSH IRMA (mU/l) 20 min
Ref. Range			60-145	1.1-2.7	10-22.5	4.0-7.8	<5.7	-	0.14-5.9	-
1	280	0	197	3.8	45	15.4	<1.0	<1.0	<0.1	<0.1
		12	29	0.8	4	2.3	109	-	115	>267
2	280	0	269	8.0	101	36.4	<0.8	<0.8	<0.1	<0.1
		8	26	0.9	4	2.0	15.0	-	17.1	37.2
3	600	0	177	4.7	68	23.0	<0.9	<0.9	<0.1	<0.1
		8	<20	0.5	1	1.0	128	-	>267	>267
4	280	0	266	5.0	117	30.0	<1.0	<1.0	<0.1	<0.1
		8	36	0.5	5	1.9	0.9	0.9	<0.1	<0.1
5	600	0	178	3.5	34	14.0	<0.9	<0.9	<0.1	<0.1
		4	57	0.8	7	2.8	2.3	6.7	2.6	7.5
6	280	0	294	6.6	>120	31.0	<0.9	<0.9	<0.1	<0.1
		8	53	0.6	8	2.4	<0.9	<0.9	<0.1	0.2
7	600	0	162	3.3	34	16.0	<0.8	<0.8	<0.1	<0.1
		8	42	0.9	6	2.4	183	-	185	>244
8	280	0	246	5.7	71	26.0	<1.0	<1.0	<0.1	<0.1
		12	<23	0.6	2	1.2	94.6	-	107	125
9	400	0	300	4.0	87	28.0	<0.9	<0.9	<0.1	<0.1
		12	136	2.5	21	6.4	<1.0	<1.0	<0.1	<0.1
10	600	0	163	3.1	31	8.7	<0.9	1.3	<0.1	<0.1
		12	74	1.7	13	4.7	<1.0	<1.0	<0.1	<0.1
11	CBZ	0	228	5.6	50	19.3	<0.9	<0.9	<0.1	<0.1
		12	118	2.1	18	6.9	1.3	1.3	0.15	1.1
12	1200	0	172	2.9	35	13.7	<1.0	<1.0	<0.1	<0.1
		12	137	2.4	19	6.2	<0.9	<0.9	<0.1	<0.1
13	320	0	159	3.5	44	12.8	<1.0	<1.0	<0.1	<0.1
		12	111	2.4	27	9.4	<1.0	<1.0	<0.1	<0.1

*Patients became either hypothyroid (1-8) euthyroid (9-12) or remained hyperthyroid (13), after therapy. Results 12 weeks post-therapy or at the onset of hypothyroidism are shown with the pre-treatment values.

Thyrotroph recovery indicated by a TSH increment (RIA or IRMA) greater than 1 mU/l after TRH was demonstrated in these 8 patients at 4, 8 and 12 weeks in 1, 3 and 4 patients, respectively.

In three of those who became hypothyroid, a small increment (<1mU/l) in TSH after TRH was detected by IRMA but not the RIA (Table 7.2). The patient treated with carbimazole (patient 11) also had a detectable basal TSH concentration by IRMA.

Table 7.2 Thyroid Hormones and TRH Tests in Four Patients after Treatment for Thyrotoxicosis

	Patient			
	1	6	7	11
Treatment	^{131}I	^{131}I	^{131}I	CBZ
Weeks post-treatment	8	8	4	12
Total T_4 (60-145 nmol/l)	94	53	108	118
Total T_3 (1.1-2.7 nmol/l)	1.1	0.6	1.8	2.1
fT_4 (10-22.5 pmol/l)	16	8	15	18
fT_3 (4.0-7.8 pmol/l)	4.4	2.4	5.9	6.9
TSH RIA (mU/l)				
0 min (<5.7)	<0.8	<0.9	1.4	1.3
20 min post TRH	<0.8	<0.9	1.4	1.3
TSH IRMA (mU/l)				
0 min (0.14-5.9)	<0.1	<0.1	<0.1	0.15
20 min post TRH	0.3	0.2	0.3	1.1

CBZ = Carbimazole

A small TSH response to TRH may, therefore, be detected by IRMA but not RIA in patients treated for thyrotoxicosis, representing partial thyrotroph recovery.

7.2 THE RELATIONSHIP BETWEEN PITUITARY AND OTHER TARGET ORGAN RESPONSIVENESS IN HYPOTHYROID PATIENTS RECEIVING THYROXINE REPLACEMENT

Many patients receiving T_4 replacement have serum TSH levels undetectable by sensitive assay (Wehmann et al., 1983; Semple et al., 1985) consistent with their absent TSH responses to TRH (Evered et al., 1973). However, the pituitary differs from some other tissues in that T_3 derived from local conversion from T_4 within the cell occupies a greater proportion of T_3 nuclear receptors than T_3 derived from serum (Larsen, 1982). This greater sensitivity of the pituitary to serum T_4 has led to the assumption that it is an unrepresentative tissue regarding the assessment of over-treatment with T_4. In this section, I have investigated whether thyrotroph suppression may be used to assess over-replacement in hypothyroid patients receiving T_4.

7.2.1 Biochemical Markers of Thyroid Status

Some constituents of serum which can be measured easily are known to alter in patients with overt thyroid disease. High concentrations of ALT, (Ashkar et al., 1971), liver-specific GST (B_1B_1) (Beckett et al., 1985), SHBG (Anderson, 1974) and ACE (Smallridge et al., 1983)

have all been described in hyperthyroidism, whereas concentrations of TBG and creatinine may be decreased (Burr et al., 1977; Bradley et al., 1974). Raised concentrations of CK are found in hypothyroidism (Doran & Wilkinson, 1975). Levels of GST and ACE in sera from patients attending a thyroid clinic (Section 4.1) are shown in Figure 7.1, illustrating the higher values found in hyperthyroid patients. Changes in the concentrations of the above analytes reflect altered entry to (due to changes in synthesis or membrane permeability) or clearance from, the blood. In the following study, in addition to measurements of total and free thyroid hormones (Amerlex) and basal TSH IRMA (Boots-Celltech), measurements of GST (B_1B_1), ALT, GGT, Creatinine, SHBG, TBG, CK and ACE were made to assess the suitability of the T4 replacement dose in hypothyroid patients.

7.2.2 Patients Studied

Two groups of patients were studied. Group I consisted of 21 hypothyroid patients (17 women, 4 men; mean age 50 years, range 31-68) starting T4 replacement. The causes of hypothyroidism in this group were: primary atrophic disease (n=7), Hashimoto's thyroiditis (n=2), and following radioiodine therapy for either Graves' disease (n=11) or multinodular goitre (n=1). The average length of time between radioiodine treatment and the start of T4 replacement was 5 months (range, 2-11). The T4

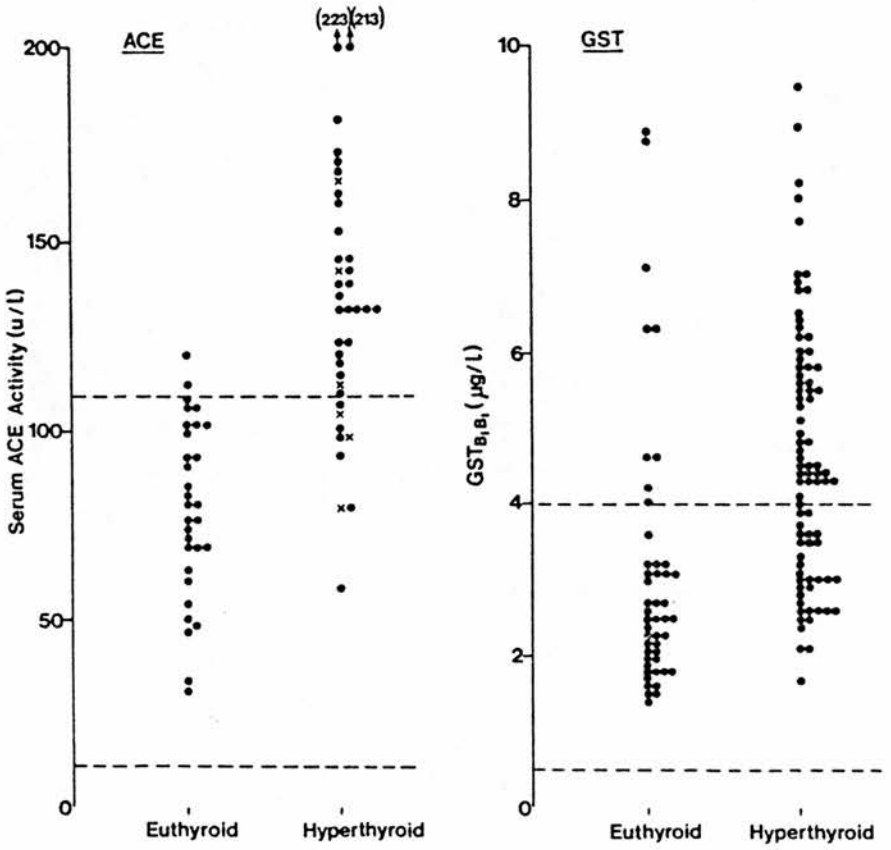


Figure 7.1 Concentrations of ACE and GST in Serum from Euthyroid and Hyperthyroid Patients.

Levels were significantly higher (Mann-Whitney, $p<0.001$) in the hyperthyroid patients. Levels of ACE in patients with toxic nodules or multinodular goitres are as indicated (x).

dose was increased each month by 50 μg increments to a maximum of 200 μg/day. Blood samples were taken at the end of each month. In addition, 5 group I patients had samples taken pre- and post-radioiodine treatment before T_4 therapy was started.

To address the question of whether equilibration of serum analytes had occurred in 1 month, nine patients from group I were re-tested after 2 additional months of T_4-treatment with the highest dose that each patient had tolerated during the study.

Group II comprised 104 patients (91 women, 13 men; mean age 51 years, range 17-85). Hypothyroidism was the result of radioiodine or surgical treatment of thyrotoxicosis in 51 and 9 patients, respectively, and in the remainder it was due to Hashimoto's thyroiditis (23 patients) or primary atrophic disease (21 patients). At the time of investigation, all were clinically euthyroid and had been receiving a constant replacement dose of T_4 for at least 3 months (mean, 3.5 years), the majority (>89%) had taken the same dose for more than 6 months. The largest group (n=47) were taking 100 μg/day, the others were taking 50 μg (n=5), 75 μg (n=1), 150 μg (n=28), 200 μg (n=19) or 300 μg (n=4) per day.

7.2.3 Patients Taking Increasing Doses of Thyroxine (Group I)

Thyroid function tests: serum total and free T_4 and T_3 concentrations were restored to normal at a lower dose of

T_4 than that necessary to suppress TSH to within the reference range and 10 patients with increased fT_4 levels had normal TSH concentrations (Figure 7.2). Normal TSH results were achieved in 18 patients at the 50, 100, 150 and 200 µg dose in 1, 8, 8 and 1 patients, respectively. Four patients could not tolerate the 200 µg/day dose for 4 weeks. Serum TSH became undetectable (<0.1 mU/l) in 17 patients; this first occurred at the 100, 150 and 200 µg doses in 1, 7 and 9 patients, respectively. Serum fT_4 was above normal in all patients when TSH first became undetectable whereas in most patients serum total T_4, total T_3 and fT_3 levels were normal at that time. Conversely, if serum total T_4, total T_3 or fT_3 were raised TSH was always undetectable.

Serum markers from peripheral tissues: dose-dependent increases (Wilcoxon matched-pairs; $p<0.05$) in GST, SHBG and ACE occurred (Figure 7.3), and levels of ALT at the 200 µg/day dose were higher than those at the 100 µg/day dose ($p<0.05$); GGT increased slightly but not significantly. Significant reductions in TBG, CK and creatinine also occurred with increasing T_4 dose (Figure 7.3).

Levels of one or more of these markers became abnormal in a total of 15 patients. Abnormally high GST, SHBG, ALT, GGT and ACE levels were found in 10, 7, 4, 3 and 3 patients, respectively. The occurrence of these

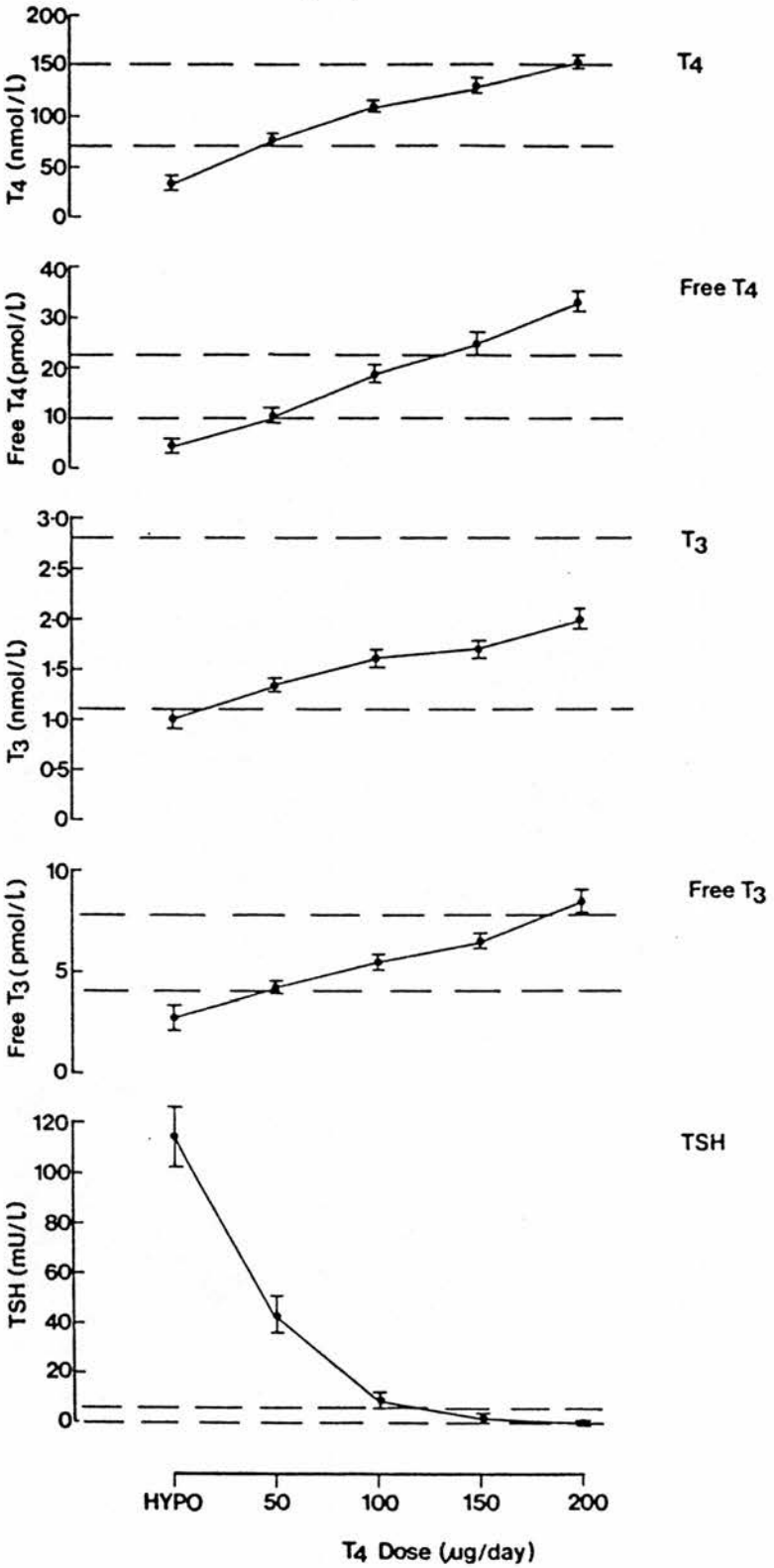


Figure 7.2 Thyroid Function Test Results in 21 Hypothyroid Patients Receiving Increasing Doses of Thyroxine at 4-week Intervals (Mean±SEM).

Reference ranges are indicated by the dashed lines.

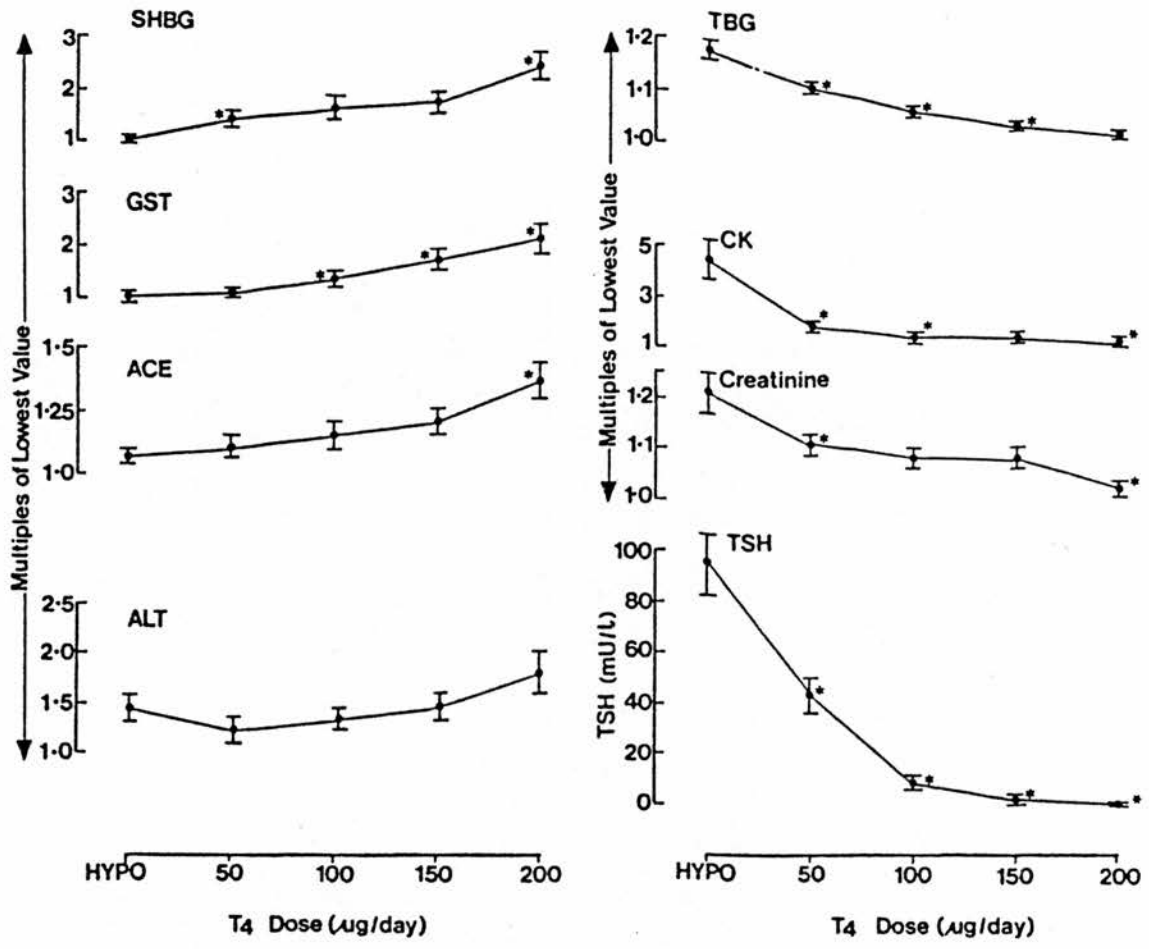


Figure 7.3 Peripheral Tissue Markers Measured in Serum from 21 Hypothyroid Patients Receiving Increasing Thyroxine Doses at 4-week Intervals (Mean±SEM).

*Significant differences from the previous dose ($p<0.05$).

abnormalities coincided with an undetectable TSH and raised fT4 concentration in 9 patients, and in 4 further patients TSH was undetectable and fT4 raised after the next increment in T4 dose. By contrast, even at the highest dose, fT_3 and total T4 and T_3 were raised in only 8, 6 and 1 of these 15 patients, respectively.

In the nine patients who were studied after an additional two months, no further changes occurred in the measured thyroid and tissue parameters, except for a slight increase ($p<0.05$) in SHBG.

7.2.4 Changes in Tissue Markers in Thyrotoxic Patients after Radioiodine Therapy

The changes in thyroid function tests and tissue markers in 5 patients prior to radioiodine treatment and during subsequent T4 replacement are shown in Figures 7.4 and 7.5, respectively. The levels of tissue markers at the maximum T4 dose were not as abnormal as when patients were thyrotoxic. However, as in group I, there were trends towards higher SHBG, GST, ACE and lower TBG, CK and creatinine levels when these patients were taking 200 µg/day T4 compared to the lower doses. Only one patient in this subgroup had levels outside reference limits. Values for tissue markers in the 5 thyrotoxic patients are compared with those in the 21 group I patients (when taking the maximum dose of T4 tolerated) and the reference ranges in Table 7.3.

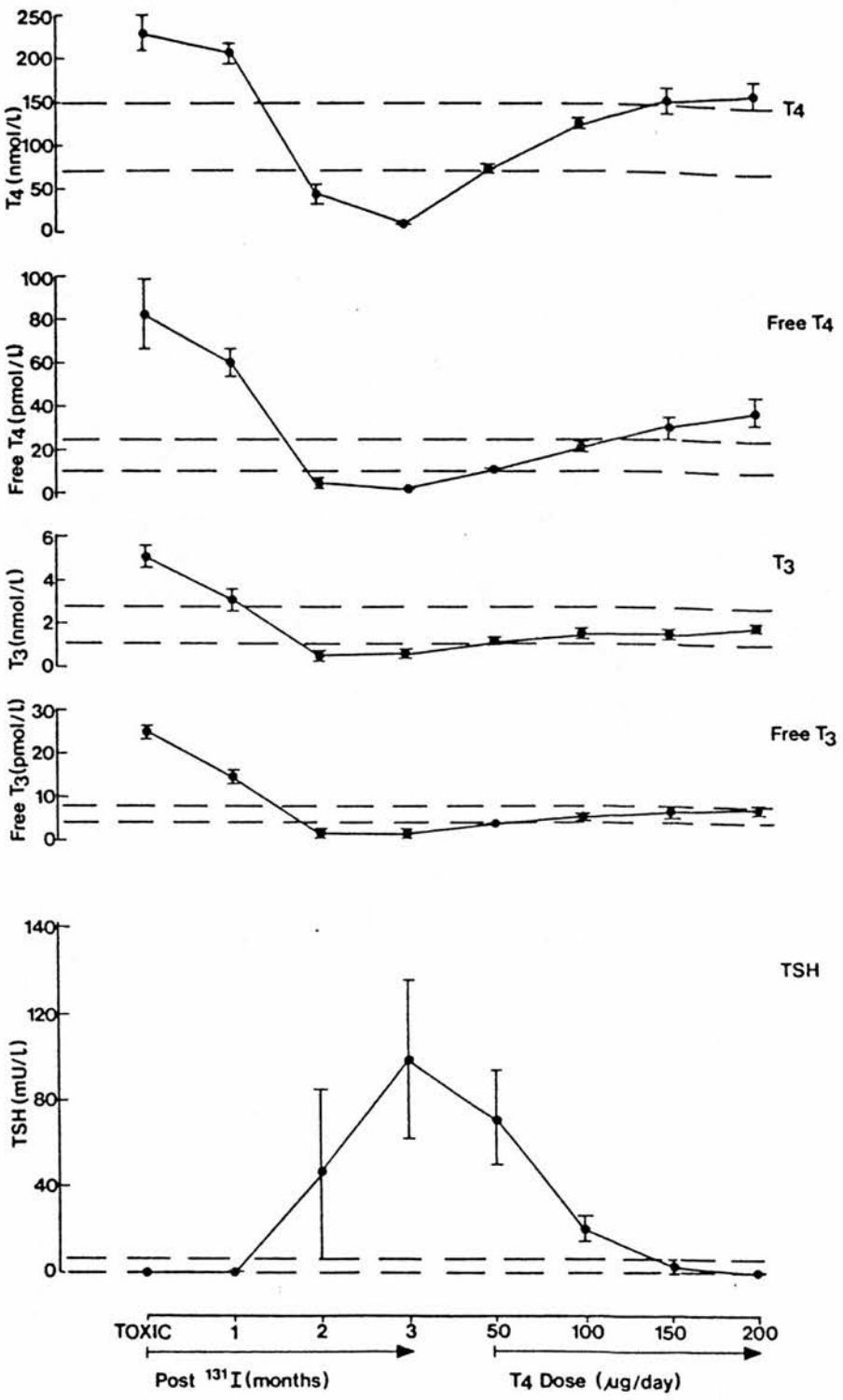


Figure 7.4 Thyroid Function Test Results (Mean±SEM) in 5 Patients Who Became Hypothyroid After Radioiodine Treatment for Thyrotoxicosis.

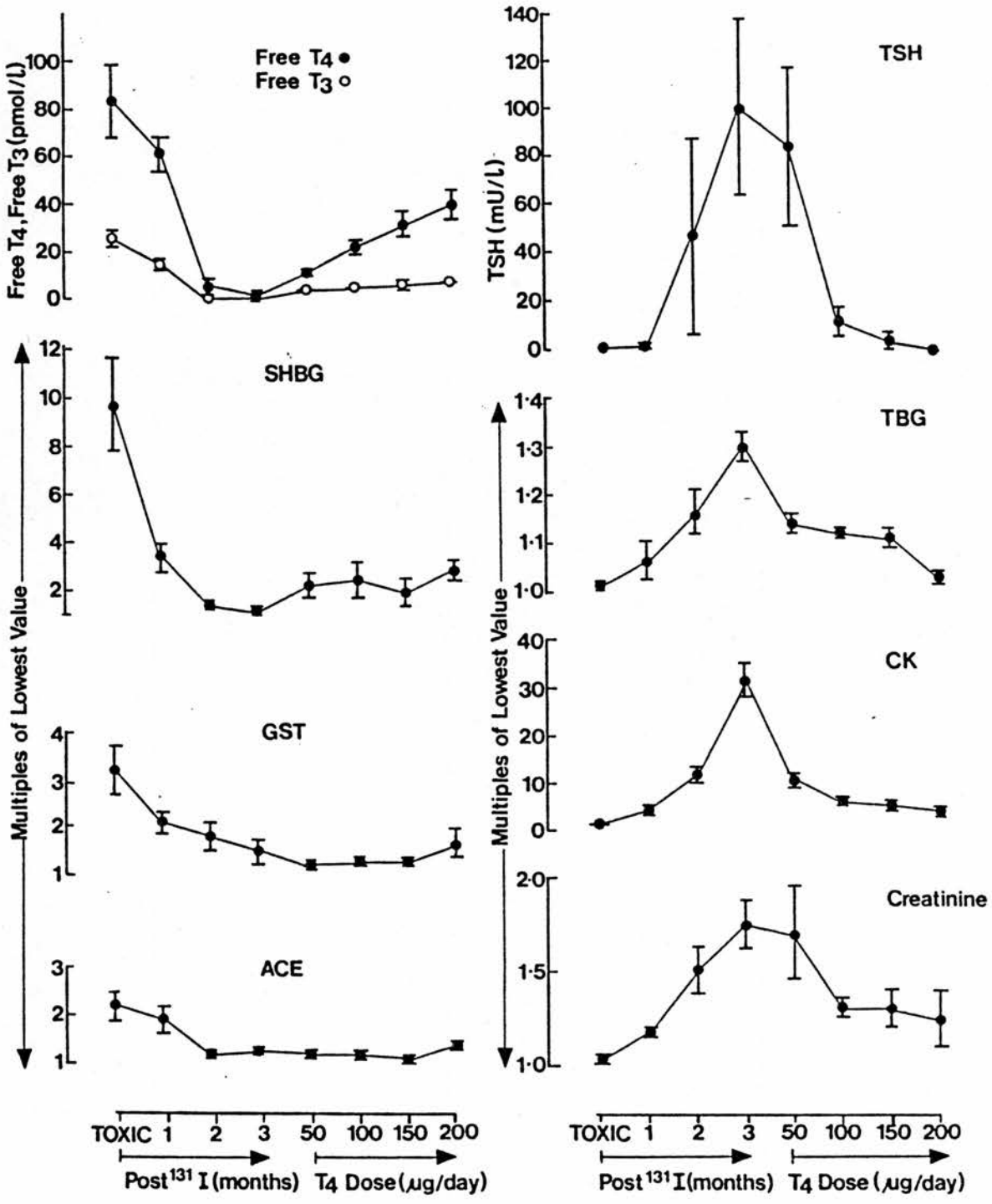


Figure 7.5 Peripheral Tissue Markers in Serum from 5 Patients Pre- and Post-radioiodine Treatment for Graves' Disease and During Thyroxine Therapy (Mean±SEM).

Table 7.3 Concentrations of Peripheral Tissue Markers in Serum from Thyrotoxic Patients (n=5) and Group I Patients (n=21)

Marker	Reference range	Thyrotoxic patients	Group I* patients
GST, µg/l	0.5-4.0	5.9± 2.7	4.8± 2.5
SHBG, nmol/l	30-90	173±50	70±33
ALT, U/l	10-40	ND	28±14
GGT, U/l	5-35	ND	21±21
ACE, U/l	11-109	129±22	83±22
TBG, mg/l	10-42	20± 3	21± 3
Creat., µmol/l	55-150	63± 9	82±16
CK, U/l	30-150	12± 9	44±26

*At maximum tolerated T_4 dose. ND = Not determined.

7.2.5 Patients Stabilised on a Fixed Dose of Thyroxine (Group II)

Thyroid function tests: measurement of TSH divided the 104 patients taking a constant dose of T_4 into 3 groups; 37 patients with undetectable TSH levels (A), 42 with normal TSH levels (B) and 25 patients with raised levels (C), Table 7.4. Only one patient had a detectable TSH concentration (0.13 mU/l) which was less than the absolute range (0.14-5.9 mU/l) found in the reference euthyroid population (Section 4.6.1). This patient was included in Group B. There was no bias towards a particular cause of the hypothyroidism between the groups or the duration of

Table 7.4 Comparison of Patient Details and Thyroid Function Tests in Group II Patients Subdivided According to Basal Serum TSH Concentration

Subgroup	Mean (SD or range)		
	A (low TSH)	B (normal TSH)	C (high TSH)
n	37	42	25
T_4 Dose (µg)	162 (60)[a]	127 (43)	116 (47)
Age (yrs)	53 (13)	53 (14)	49 (14)
Weight (Kg)	66 (14)	69 (13)	72 (12)
Duration (yrs)	3.2 (0.3-15)	3.8 (0.3-22)	3.7 (0.5-21)
TSH (mU/l)	<0.1[b]	1.4 (0.13-5.9)	24.3 (6.4-103)[b]
fT_4 (pmol/l)	35.0 (17.5)[b]	21.2 (3.7)	15.0 (5.0)[b]
fT_3 (pmol/l)	7.1 (2.8)[b]	5.7 (1.4)	4.3 (1.4)[c]
Total T_4 (nmol/l)	148.7 (43.8)[b]	118.8 (20.0)	92.5 (28.7)[b]
Total T_3 (nmol/l)	1.9 (0.4)[b]	1.6 (0.3)	1.4 (0.3)

Differences from group B; $p<0.005^a$, $p<0.001^b$, $p<0.05^c$

Reference ranges: TSH, 0.14-5.9 mU/l; fT_4, 10.0-22.5 pmol/l; fT_3, 4.0-7.8 pmol/l; total T_4, 60-145 nmol/l; Total T_3, 1.1-2.7 nmol/l.

therapy, but patients in group A were taking higher doses of T_4 than those in group B. From Table 7.4, a mean reduction of 22% in the dose of T_4 would bring group A patients to the same mean dose as those in group B. Patients in group C had not been prescribed less T_4 than group B, but 7 were thought to be non-compliant with their treatment. The prescribed dose did not correlate significantly with the patient's age or weight. For detectable TSH values, the Spearman's correlation coefficients ($p<0.0001$) for TSH versus free thyroid hormones were $r = -0.746$ for fT_4 (Figure 7.6) and $r = -0.446$ for fT_3.

In group A; fT_4, total T_4, fT_3 and total T_3 were high in 32, 13, 12 and 1 patient, respectively. In group B, high fT_4 levels were found in 14 patients and high total T_4 levels were found in 3. One patient in group B had low total T_4 and fT_3 results, and low levels of thyroid hormones were found in 7 patients from group C.

Serum markers from peripheral tissues: the results for serum ALT, GST, GGT, CK, TBG and SHBG are shown for the three groups in Figure 7.7. For the TBG and SHBG data, women taking an OCP and all men were excluded. By the Mann-Whitney test, significantly higher ALT ($p<0.001$), GST ($p<0.02$) and GGT ($p<0.05$) levels were found in group A than in group B. Group A had lower CK levels than group B ($p<0.05$). Compared with group C, group A

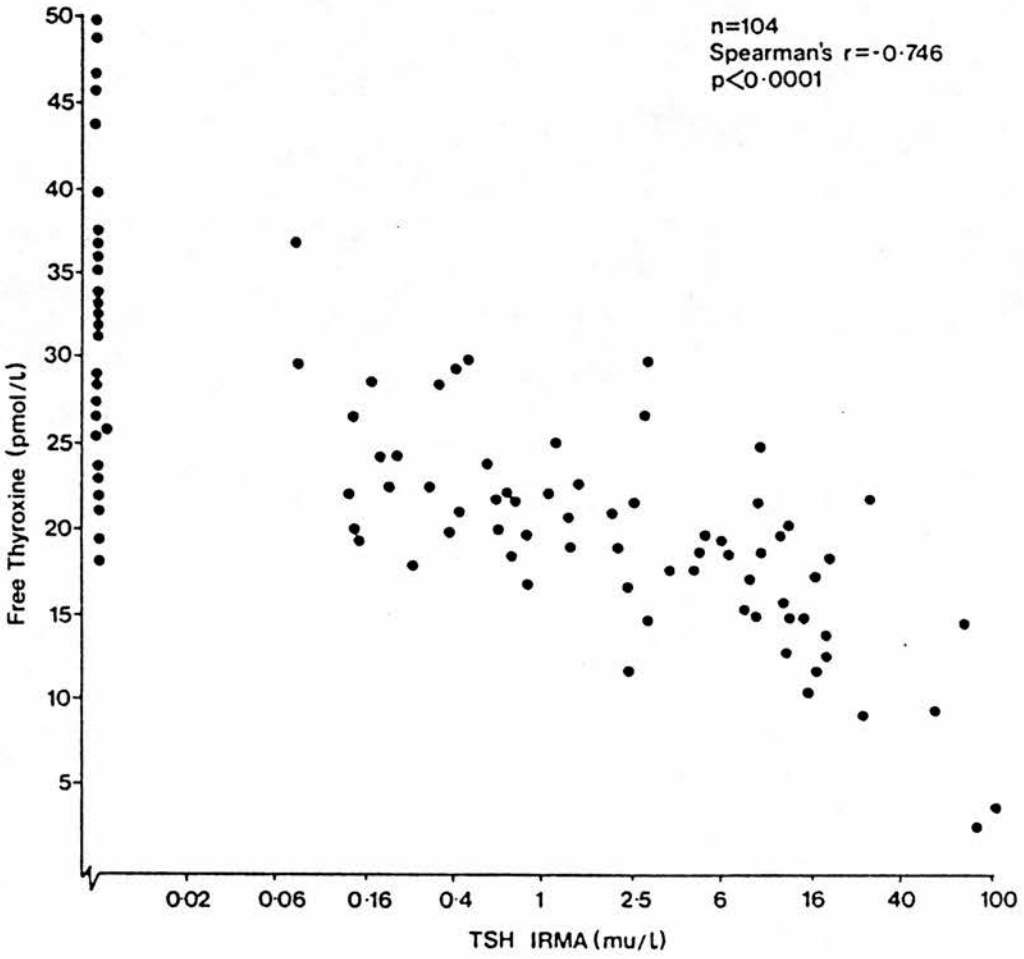


Figure 7.6 The Spearman's Correlation between Serum Free T_4 and Basal TSH (IRMA) in Patients Taking Thyroxine Replacement.

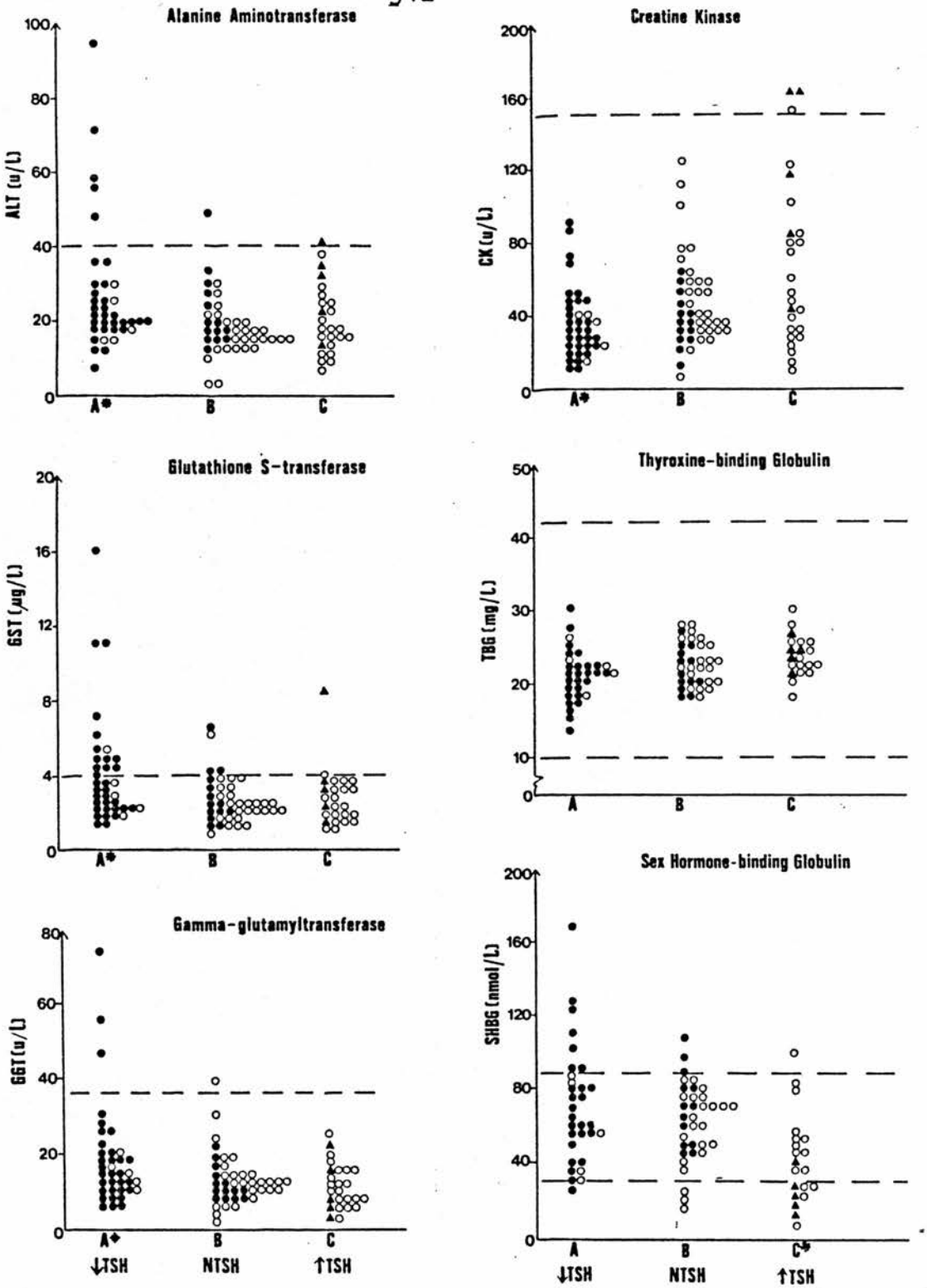


Figure 7.7 Peripheral Tissue Markers in Serum from 104 Patients Taking a Fixed Dose of Thyroxine Grouped According to TSH (IRMA) Levels.

*Significant differences from Group B ($p<0.05$). Patients with high (●), normal (o) and low (▲) fT_4 levels are shown.

had higher SHBG ($p<0.001$) and lower TBG levels ($p<0.02$). ACE activities and creatinine levels did not differ significantly between the groups.

A total of 27 patients had an abnormally high level of at least one of the following; ALT, GST, GGT, SHBG. Most of these patients had an undetectable TSH (67%) or raised fT_4 (85%), whereas fT_3 was raised in only 33% and total T_4 raised in 26%, respectively. Total T_3 concentrations were within reference limits in all 27 patients.

7.2.6 Thyroid Function Tests and Tissue Markers after a Reduction in Thyroxine Dose in Group II Patients

Thirteen patients from Group II who had undetectable TSH (IRMA) and raised fT_4 levels and who lived locally, were recalled for the performance of a TRH test and a reduction in their dosage by 25-100 µg. All patients had a TSH response less than 1 mU/l after TRH by RIA and IRMA before the dose was reduced (Table 7.5). Basal TSH IRMA was $\leqslant 0.1$ mU/l in all but one patient whose value was 0.4 mU/l with an increment of 0.9 mU/l after TRH. Measurements of peripheral tissue markers, thyroid hormones and TRH tests were performed at two-month intervals until increments in TSH after TRH, by RIA and IRMA, were greater than 1 mU/l in all patients. At the lower dose, values for total T_4, T_3, fT_4 and fT_3 became abnormally low in 0, 1, 1 and 2 patients, respectively. A significant

Table 7.5 Thyroid Hormones, Thyrotroph Responsiveness and Tissue Marker Concentrations after a Reduction in Thyroxine Dose in 13 Group II Patients (Mean±SD)

	Thyroxine Dose*			
	A	B	Significance (P)	
			t-test	Wilcoxon test
Dose (µg/day)	164.3 (41.3)	112.5 (36.4)	<0.0001	<0.0001
Weight (Kg)	68.2 (14.7)	68.1 (14.8)	NS	NS
Total T_4 (60-145 nmol/l)	143.9 (22.6)	117.5 (21.8)	<0.005	<0.005
Total T_3 (1.1-2.7 nmol/l)	1.5 (0.3)	1.4 (0.3)	<0.05	<0.05
fT_4 (10-22.5 pmol/l)	25.1 (4.6)	18.8 (4.9)	<0.0001	<0.005
fT_3 (4.0-7.8 pmol/l)	5.8 (1.3)	4.9 (1.3)	<0.01	<0.05
TSH RIA Increment after TRH (>1 mU/l)	0.07(0.13)	6.5 (6.9)	<0.005	<0.005
Basal TSH IRMA (0.14-5.9 mU/l)	0.05(0.11)	1.9 (1.6)	<0.005	<0.005
TSH IRMA Increment after TRH	0.32(0.35)	7.9 (8.5)	<0.01	<0.005
GST (0.5- 4.0 µg/l)	4.6 (1.8)	3.4 (1.4)	<0.01	<0.02
ALT (10- 40 U/l)	22.9 (11.1)	23.1 (10.0)	NS	NS
GGT (5- 35 U/l)	20.1 (15.2)	18.3 (7.0)	NS	NS
Creatinine (55-150 µmol/l)	81.7 (32.0)	85.4 (27.8)	NS	NS
SHBG (30- 90 nmol/l)	61.5 (35.9)	54.3 (25.4)	0.13	0.09
Sleeping heart rate (beats/min)	64.7 (7.5)	61.9 (5.6)	NS	0.10

*Patients were stabilised on the lower dose (B) for at least 6 weeks before re-testing. Data were analysed by paired t-test and the Wilcoxon matched-pairs test.

change in tissue markers was discernible only for GST; values in 6 out of 8 patients with raised GST levels were reduced to reference levels after reduction in the dose of T_4 (Figure 7.8). The fall in SHBG concentrations did not reach statistical significance (Table 7.5). The patient whose GST concentration became increased further above normal after the reduction in T_4 (Figure 7.8) also had an abnormal ALT concentration before (50 U/l) and after the dose reduction (52 U/l).

7.2.7 Summary and Discussion

A relationship was demonstrated between the serum markers used to assess thyroid status in peripheral tissues and TSH levels by sensitive assay in patients taking T_4. Although the changes in SHBG, TBG, CK and creatinine may reflect the return of peripheral tissues to normal from the hypothyroid state, some patients had abnormally high ALT, GST, GGT, SHBG and ACE levels at the higher doses of T_4. The majority of abnormalities were found for GST. Although GST measurements correlate with ALT concentrations (Figure 7.9) they provide a more sensitive index of hepatocellular damage than ALT (Beckett & Hayes, 1984). These increased GST levels are not a result of enzyme induction but reflect hepatic release (Beckett *et al.*, 1986).

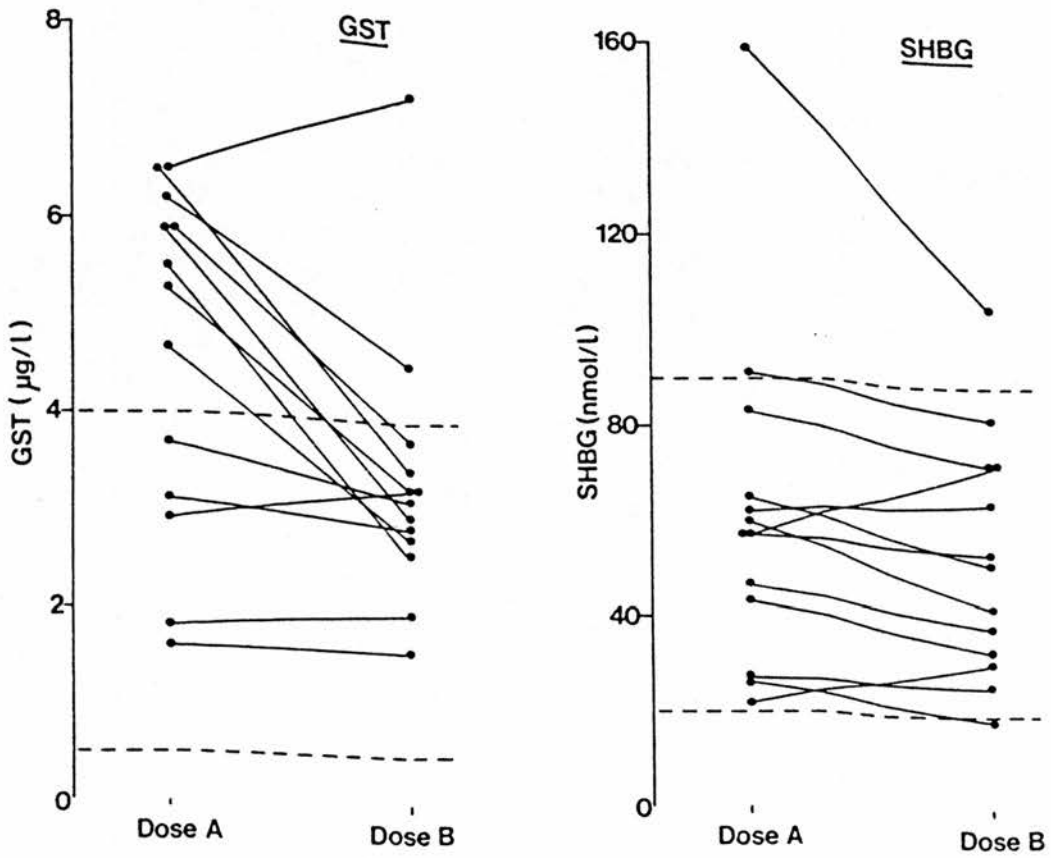


Figure 7.8 Concentrations of GST and SHBG in Serum Before and After a Reduction of Thyroxine Dose in Hypothyroid Patients.

With dose A, all patients had an absent TSH response to TRH; with dose B, responses to TRH were normal.

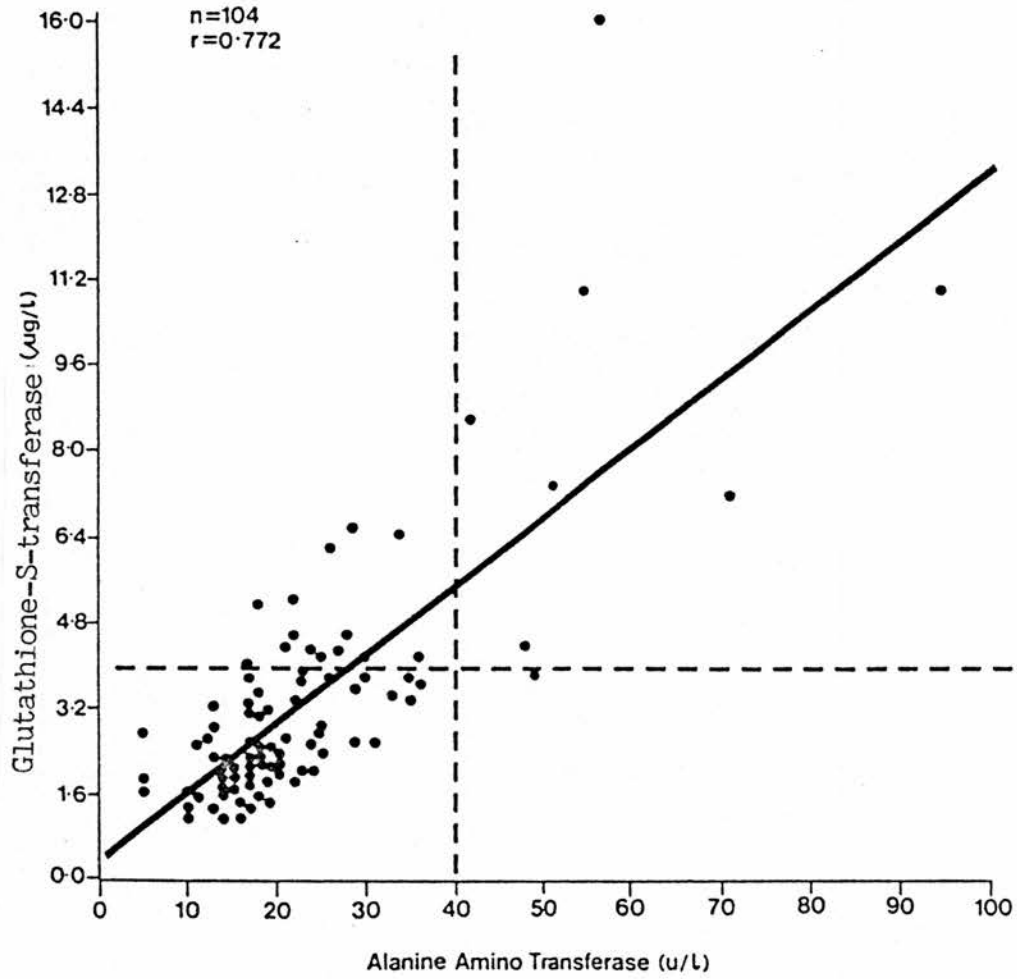


Figure 7.9 The Correlation between Hepatic GST Concentrations and ALT Levels in the Serum of Group II Patients.

The spectrum of abnormalities found in tissue markers was not as great as those found in patients with untreated overt hyperthyroidism but, in addition to the complete suppression of TSH secretion, they provide evidence of a generalised tissue over-exposure to thyroid hormones. The lack of complete concordance in the abnormalities detected and pituitary suppression is not unexpected in view of the wide range of sensitivities and specificities to thyroid status of the tissue markers measured. The results presented suggest that TSH secretion can be used as a sensitive and representative test of peripheral tissue exposure to thyroid hormones in patients taking T_4 replacement therapy. In addition to the biochemical abnormalities demonstrated in those with undetectable TSH (IRMA), such patients also tended to have lower body weights and higher sleeping heart rates than those with normal TSH (IRMA), although these differences did not reach statistical significance.

Many patients with normal serum total and free T_3 concentrations had evidence of tissue hyperthyroidism suggesting that such measurements are insensitive tests of overtreatment. Although a greater proportion of patients with evidence of tissue hyperthyroidism had raised fT_4 concentrations than an undetectable TSH (IRMA) a substantial proportion of patients required a high fT_4 level in order to reduce TSH secretion to normal. Adjustment

of T_4 dosage to normalise fT_4 values might, therefore, lead to problems of under-replacement. Reduction of T_4 dosage to give a detectable TSH (IRMA) level in 13 patients resulted in normal TSH responsiveness to TRH and a significant reduction in the number of patients with abnormal GST concentrations. Total and free thyroid hormone concentrations remained within reference limits in the majority of patients after the reduction in T_4 dosage, indicating sufficient replacement for other tissues.

Thus the data presented suggest that the object of T_4 replacement therapy should be to achieve detectable TSH (IRMA) concentrations within the reference range.

Chapter 8

DISCUSSION

General aspects of this thesis are discussed under three main headings in this chapter. The analytical validity and physiological relevance of the new thyroid function tests are discussed initially followed by an assessment of their clinical value and finally their role in new test strategies.

8.1 THE VALIDITY AND RELEVANCE OF FREE THYROID HORMONE AND SENSITIVE TSH MEASUREMENTS

8.1.1 Analytical Validity

There has been much recent debate about the analytical validity of both the new analogue RIA methods and the purported reference method of equilibrium dialysis for measuring free thyroid hormones in serum. This is discussed below followed by an assessment of the analytical validity of sensitive TSH measurements.

(a) Equilibrium Dialysis Methods

The direct dialysis methods developed in this thesis for fT_4 and fT_3 compared very well with other reference methods in that they produced similar reference ranges to other reports and, in addition, the biases of fT_4 kit methods compared to dialysis were in agreement with results found in a recent DHSS survey (Chapter 3). Dialysis results were also unaffected by serum dilution using out-patient samples (Chapter 3) and independent of changes in the concentration and affinity of serum binding proteins (Chapter 5), thus fulfilling the major criteria for analytical validity of a free hormone measurement.

Some authors, however, have questioned whether direct dialysis is suitable as a reference technique since it is cumbersome, relatively imprecise and may be particularly susceptible to the presence of weak binding-protein inhibitors in serum (Wilkins et al., 1982 & 1985). All methods which result in serum dilution will give incorrect results when such inhibitors are present. Ultrafiltration of undiluted serum or equilibrium dialysis against a small dialysate volume are therefore required for valid measurements in this situation. However, it has been argued that any non-dialysable inhibitor (e.g. NEFA) will produce artificial elevation in fT4 measured by dialysis because a concentration gradient of the inhibitor is set up across the membrane which must be balanced thermodynamically by an opposite gradient of T4 (Wilkins et al., 1982 & 1985). This explanation was proposed to account for major differences between dialysis results and those found by analogue RIA methods in certain circumstances but no experimental evidence or formal scientific basis has been presented to support it; Jackson & Ekins (1986) have concluded that it is the analogue RIA methods which are analytically invalid in this situation. In support of dialysis methods, it has been shown that high NEFA concentrations in vitro and in vivo also produce high fT4 values by non-analogue two-step RIA (Bayer, 1983b) and by ultrafiltration methods (Wang et al., 1986).

In some patient samples, the NEFA concentration may increase *in vitro* due to continued activity of lipoprotein lipase and it is argued that the overnight incubations at 37°C used in equilibrium dialysis will accelerate this process leading to spuriously high results. Higher fT_4 values by dialysis have been reported compared to ultrafiltration (Wang *et al.*, 1986) and the Clinical Assays two-step method (Bayer, 1983b) in patients receiving heparin, providing some evidence for this effect. However, this does not appear to be a general problem since there is a high degree of correlation in thyroid clinic samples between dialysis values and those by analogue RIA (Chapter 4; Liewendahl *et al.*, 1986) and by non-analogue kit methods (Jackson & Ekins, 1986).

In conclusion, since the equilibrium dialysis and ultrafiltration techniques have a sound theoretical basis and their limitations are well recognised, to date they remain the most valid reference methods for free thyroid hormone measurement.

(b) Free T_4 and Free T_3 by Analogue RIA

Several major reservations have been expressed as to the validity of analogue RIA methods centering on theoretical arguments and experimental findings which have primarily been directed at the Amerlex fT_4 system since this was the first analogue RIA developed (Stockigt *et al.*, 1982a & 1983; Ekins *et al.*, 1983 & 1984; Wilke, 1984; Bayer, 1983a & 1984; Jackson & Ekins, 1986).

These criticisms are:-

1. A significant proportion of the analogue is bound to serum proteins during the RIA.
2. The antiserum binds a greater proportion of the total hormone pool than is theoretically acceptable for a valid fT_4 measurement.
3. Since the serum is diluted in the RIA, analogue assays will not measure fT_4 accurately if weakly-bound inhibitors of thyroid hormone binding are present in serum.

The labelled analogue method was originally presented as relying on the analogue being essentially unbound to serum proteins. However, Ekins et al. (1983) and others have calculated from the amount of antibody used in the assay that about 98% of the analogue is in fact bound to serum proteins since otherwise too high a proportion of the total T_4 pool would be sampled by the antibody leading to perturbation of the binding equilibrium. It is now acknowledged by the manufacturers that some residual binding of the analogue to serum proteins is necessary for measurement of fT_4 by analogue RIA (Midgley & Wilkins, 1983; Wilkins et al., 1985). This residual binding could explain why free hormone values (a) decrease dramatically with serum dilution (Chapter 6), (b) correlate significantly with the serum albumin level in pregnancy (Chapter 5), NTI (Chapter 6) and in analbum-

inemia (Stockigt et al., 1983), (c) increase proportionately with added albumin in vitro (Chapter 5), (d) are spuriously high in the presence of abnormal high-affinity binding proteins (Chapter 5) and (e) decrease when inhibitors (e.g. oleic acid, salicylate) alter the usual analogue binding to serum proteins (Chapter 6; Wilke, 1986). In the above situations, the amount of analogue available to bind to the assay antibody may change significantly relative to the kit standards: when binding to serum protein is abnormally high, less of the analogue may take part in the RIA and the converse may occur when serum protein binding is low or when binding inhibitors (e.g. NEFA) are present. In spite of the above findings, the proponents of the analogue RIA concept argue that the binding of the analogue to serum proteins is insufficient to distort Amerlex fT_4 values in most patients (Wilkins et al., 1985; Wilkins & Midgley, 1986). Their analogue binds weakly to TBG and TBPA at 2-3%, and to albumin at 12-30% of the affinity of T_4, and it is maintained that in the presence of antiserum, all of the analogue is readily available to participate in the RIA (Midgley & Wilkins, 1983). The distribution of the analogue binding is therefore relatively insensitive to changes in TBG and TBPA and distortion of fT_4 results is only thought likely in rare cases of variant albumin, high-titre anti-T_4 antibodies, analbuminaemia or when NEFA concentrations are massively

increased. The manufacturers also conclude that only in the latter two circumstances will greater than 5% of the total T_4 pool be sampled by the assay antibody (Wilkins et al., 1985).

Although some of the above criticisms have been largely theoretical and may not necessarily compromise the clinical value of all analogue assays in all situations, it has been suggested that these assays are unsuitable as routine tests of thyroid status and should be removed from laboratory practice (Wenzel et al., 1985; Alexander 1986; Jackson & Ekins, 1986). Attention has therefore focused on the structure of the analogues to minimise effects due to alterations in the serum binding proteins (Geiseler et al., 1986). Further modifications of the analogues to achieve this, however, reduce their similarity to T_4 and may significantly alter their antibody-binding characteristics. The analogue assays produced by manufacturers may therefore perform differently in various patients' sera as evidenced in this study by:-

(a) different correlations with serum albumin.

(b) different numbers of low values found in pregnancy and in NTI.

(c) different results for a patient with FDH.

(d) different biases compared to dialysis-RIA in patient samples not accounted for by differences in standardisation.

Albumin deficiency and heparin or salicylate therapy may also affect the analogue assays to different extents

(Wilke, 1984 & 1986). However, in agreement with the manufacturers, Wilke (1984) found that the albumin effect in the Amerlex fT_4 assay was not sufficiently great to invalidate its clinical utility when binding proteins in serum were either normal or "moderately" abnormal. This conclusion is supported by the excellent correlations with direct dialysis values in patients from a thyroid clinic reported in this thesis and by Liewendahl et al., 1986. The results from non-analogue kits are theoretically more valid but they involve more manipulations and are less precise (Jackson & Ekins, 1986; Csako et al., 1986). Therefore, although the analytical validity of analogue free thyroid hormone assays is questionable, particularly in certain circumstances outlined above, the question arises whether, in a routine setting, they give results of greater clinical value than total hormone measurements.

(c) Immunometric TSH Measurements

The introduction of immunometric assays for TSH has met with relatively little criticism. The presence of heterophilic (e.g. anti-mouse) antibodies in serum may, however, produce more noticeable interference in two-site assays than has been reported for RIA methods, since the cross-linking of the assay monoclonal antibodies by multivalent interfering substances may give falsely detectable TSH values (Hunter & Budd, 1980). Boscato & Stuart (1986), report the prevalence of heterophilic antibodies

in serum to be as high as 40%. However, effects may not be apparent because (a) animal serum is present in the assay reagents which saturate the binding sites of the interferent and (b) there is higher relative binding of the analyte. Isolated instances of assay interference producing falsely high TSH values (Cusick et al., 1985; Clark et al., 1985) or falsely detectable values in Graves' hyperthyroidism have been found using the Amerwell IRMA in this and other laboratories (Finlayson et al., 1987; Gow et al., 1987) and with other two-site assays (John & Henley, 1987); this interference is ameliorated in the majority of cases by the inclusion of mouse serum in the assay.

The measurement of TSH in sera from pregnant or post-menopausal women has been improved by the greater specificity of immunometric assays compared to RIA, decreasing problems with cross-reactivity (Bruce et al., 1986). However, significant negative interference due to high HCG concentrations has been demonstrated for some monoclonal combinations (Chapter 5). Differences in the potency of monoclonal combinations to bind endogenous and standard TSH may also account for systematic differences in TSH concentrations by different kits which are correctly standardised (Paterson et al., 1985; Biggart et al., 1985). Matrix effects have also proved responsible for the persistent negative bias found in one TSH IRMA (Clark et al., 1986).

There may also be divergence between immunological and biological activity of TSH (as with other peptide hormones) highlighted by the increased use of immunometric assays. Faglia et al. (1979) showed this by RIA in patients with secondary hypothyroidism and it might not be unexpected in cases of TSH-secreting tumour where structurally aberrant forms of TSH might be secreted. An increased high molecular weight TSH with impaired biological activity has been reported in a euthyroid patient (Spitz et al., 1981); two-site TSH assays might give different results to RIA in such situations.

Immunoradiometric assays offer clear advantages over RIA in terms of analytical sensitivity and working range. Samples with high TSH levels can be analysed without prior dilution and the use of coated wells and non-isotopic labels will provide speed, robustness and increased sensitivity due to the higher signals generated compared to radiolabels (Woodhead & Weeks, 1985). It remains to be seen whether increased analytical sensitivity will add significantly to the clinical value of sensitive TSH measurements by IRMA.

8.1.2 Do Measurements of Free Thyroid Hormones and TSH (IRMA) Accurately Reflect Thyroid Status?

Irrespective of the analytical validity of a hormone measurement, there is still some question as to whether the free hormone concentration is the principal determinant of the cellular action of thyroid hormone.

There is some evidence in animals to suggest that thyroid hormones may enter cells by active transport. If this is the case in man then it will detract from the current thinking concerning the importance of the free hormone fraction since both free and bound fractions would be available for entry into cells. In certain tissues e.g. the liver and placenta, theoretical and experimental models suggest that the protein-bound fraction is available to cells.

That TSH secretion may not always accurately reflect thyroid status is suggested by the fact that low and undetectable levels can occur in clinically euthyroid patients who have normal thyroid hormone concentrations in serum e.g. in patients with solitary nodules or multi-nodular goitre, patients with severe illness and patients taking T_4 replacement. Raised TSH levels have also been found in clinically euthyroid patients either taking T_4 replacement or during recovery from illness. The question of the physiological relevance of TSH and free hormone measurements in various patient groups will now be discussed.

(a) Uncomplicated Patients

In patients attending thyroid clinics the clinical and biochemical picture is not usually complicated by effects of systemic illness and drug administration. In contrast to total T_4 and T_3 measurements, free hormone

measurements do not give misleading results in TBG excess and deficiency and they therefore correlate better with the nuclear T_3 receptor content of peripheral tissue and the clinical impression of thyroid status (Chapter 5; Pearce & Byfield, 1986). They do not, however, provide the most sensitive test of mild thyroid dysfunction.

With the exclusion of pituitary disease or TSH-secreting tumours, measurements of TSH secretion correspond well with thyroid status and also detect minor degrees of thyroid dysfunction not evident from thyroid hormone results. The fact that some changes in peripheral markers of thyroid status occur in patients with mild thyroid disease (Forfar et al., 1979; Bell et al., 1983; Cooper et al., 1984) supports the premise that in uncomplicated patients, abnormal TSH secretion is a more accurate measure of thyroid status than thyroid hormone tests and clinical judgement.

(b) Pregnancy

Many studies have now shown that total thyroid hormone and free hormone values by analogue-RIA are not accurate indicators of thyroid status in pregnancy, principally because of the physiological changes in TBG and albumin concentration in serum (Chapter 5). The large decrease in analogue free hormone values with gestation cannot be ascribed simply to high NEFA concentrations in late pregnancy (Chapter 5) and the NEFA:albumin ratio is

unlikely to be high enough to significantly alter the binding of thyroid hormones to serum binding proteins.

The decrease shown in free thyroid hormone values within the reference range in pregnancy by equilibrium dialysis is in agreement with other workers (Chapter 5). Bounaud et al. (1986) also found this for fT_4 measured by column adsorption chromatography and RIA as did Kurtz et al. (1979) using a two-step back titration method. Some decrease in values has been shown by other non-analogue commercial methods (Jackson & Ekins, 1986).

Ekins (1984), has suggested that increased demand for iodine by the foetus may cause this progressive fall in free thyroid hormones. Alternatively, there may be a resetting of the maternal HPT axis to lower circulating free hormone levels (Smith & Bold, 1983). Kvetny & Poulson (1984) have shown that there is a progressive increase in the nuclear binding capacity for T_3 and T_4 in human mononuclear cells with gestation. Whether this represents compensation at the tissue level for the slightly low circulating free hormone levels or, alternatively, that free hormone levels are lowered to maintain euthyroid nuclear-receptor occupancy when there is an increase in receptor numbers during pregnancy, remains an open question. The demonstration that free thyroid hormone levels decrease in post-menopausal women receiving oestrogen therapy, has led some authors to suggest that

the rise in oestrogen level in pregnancy triggers the decrease in free hormone concentration (Abdalla et al., 1984). This effect might be mediated directly at the thyroid (Zaninovich et al., 1982) or at the cellular level in modulating cellular uptake or nuclear-receptor binding (Franklyn et al., 1983; Kvetny & Poulson, 1984).

The fact that basal TSH concentrations rise progressively within the normal range with gestation (Chapter 5) is consistent with normal negative feedback control at the pituitary. Recent evidence suggests that the decrease in free hormones could be interpreted, in part, as the return to normal values from a mild hyperthyroidism in early pregancy due to the extra stimulation of the thyroid by HCG. The borderline-high free hormone values reported in early pregancy in some longitudinal studies (Yamamoto et al., 1979; Weeke et al., 1982; Guillaume et al., 1985) together with the low TSH results by sensitive assay, may therefore be an accurate measure of thyroid status. In late pregnancy, the significance of slightly low free hormone values is not fully understood since the normal basal TSH concentrations and clinical impression suggest euthyroidism.

(c) Non-thyroidal Illness

In NTI, the total T_3 concentration in serum is often low due to a decrease in extra-thyroidal conversion of T_4 to T_3 with an increase in serum rT_3 levels. There

may also be a reduction in the TSH response to TRH. Low total T_4 concentrations also occur particularly in more severe illness and this is a poor prognostic sign (Chapter 6). The concentration of serum binding proteins may be decreased with an increase in the desialated form of TBG, which has low affinity for thyroid hormone (Reilly & Wellby, 1983). The physiological relevance of these changes is unclear and it has been assumed that free hormones are normal accounting for the normal basal TSH concentrations and clinical euthyroidism in patients with NTI. However, fT_4 measurements by direct dialysis and ultrafiltration suggest that values are often high in undiluted serum and that they decrease with serum dilution indicating the presence of an endogenous inhibitor of T_4-binding (Chopra et al., 1983; Nelson & Weiss, 1985; Boye & Weeke, 1986). Although some recent studies suggest that this is oleic acid (Chopra et al., 1985) other studies argue against the levels of oleic acid being sufficiently high in severe NTI to account for the inhibition (Mendel et al., 1986).

Confusion in the literature as to the true thyroid status in NTI has resulted from attempts to unify, in one theory, the changes which occur in the HPT axis in various different systemic diseases and in illness of different severity. In this thesis, four groups of patients were studied: general medical admissions, geriatric

patients, patients with COAD and patients with CRF. In this way, some specific disease-related changes could be identified. The alterations in thyroid function tests which occur in severely ill patients undergoing intensive care were not studied, since thyroid status is usually of minor concern in such patients. However, the findings from other studies are discussed below.

(i) General medical admissions, geriatric patients and patients with COAD

In general medical admissions (Chapter 6), dialysis fT4, NEFA, total T4 and basal TSH (IRMA) concentrations did not differ greatly from normal whereas total T_3 and dialysis fT_3 levels were low, consistent with other studies in similar patients (Kaptein, 1982; Kaplan et al., 1982; Liewendahl et al., 1984). The majority of geriatric patients and those with COAD also had normal concentrations of total T4 and TSH (IRMA), and the TSH response to TRH in general medical admissions and patients with COAD was also essentially normal. Hypoxia did not appear to have a specific effect on the HPT axis in contrast to previous reports. The patients in these groups, therefore, typify those with the low T_3 syndrome. An increase in the number of nuclear T_3 and T4 receptors in mononuclear cells has been demonstrated in insulin-dependent diabetics with the low T_3 syndrome (Kvetny, 1983). This may provide a mechanism by which euthyroidism is maintained in peripheral tissues. Low

circulating fT_3 concentrations may not, therefore, reflect decreased hormone concentration at the nuclear receptor. Measurements of the circulating fT_4 and TSH (IRMA) concentrations would appear to more accurately reflect the thyroid status of such patients.

(ii) Patients in intensive care

In studies of the thyroid status of severely ill patients, the effects of exogenous dopamine, glucocorticoids, heparin and other drugs e.g. frusemide may obscure pathophysiological changes. However, observations that TSH secretion in primary hypothyroidism is reduced in patients with coexistent, severe illness (Hooper, 1976; Slag et al., 1981b), that TSH concentrations rise on recovery (Slag et al., 1981b; Bacci et al., 1982; Hamblin et al., 1986), and that the decline in thyroid hormone levels is preceded by a decrease in TSH secretion (Wehmann et al., 1985) suggest that altered TSH secretion contributes to the low T_4 syndrome. Peripheral tissue markers of thyroid status e.g. serum ACE concentrations (Brent et al., 1984) and tissue levels of T_3 (Reichlin et al., 1973) are low in these patients and red cell Na^+/K^+-ATPase activity is high (Dasmahapatra et al., 1985), which suggests the presence of a true secondary hypothyroidism. This is likely to be an adaptive mechanism since administration of T_4 or T_3 does not improve survival and may be contra-indicated since it may produce

excessive catabolism and interfere with the timing of the recovery which appears to require return of hypothalamic-pituitary function (Brent & Hershman, 1986). Gonadotrophin secretion is also temporarily impaired indicating wider disturbance of hypothalamic-pituitary function (Quint & Kaiser, 1985; Woolf et al., 1985). These endocrine changes in critical illness are thought to be mediated by the release of endogenous stress hormones (dopamine, glucocorticoids, endorphins, somatostatin) in addition to the physiological response to fasting (Hamblin et al., 1986).

(iii) Chronic renal failure

Patients with CRF are not critically ill but invariably have low levels of total T_4 and T_3 with normal rT_3 concentrations in serum (Melmed et al., 1982; Wartofsky, 1984). Reduced responses to TRH do not appear to be due to reduced caloric intake nor increased dopamine action since there is evidence that dopamine-receptor interaction on both the thyrotroph and the lactotroph is blocked in this disease (Weetman et al., 1981). There is some inhibition of T_4-binding in this condition (Chapter 6) possibly due to metabolites e.g. phenols and indoles which accumulate in uraemia (Spaulding & Gregerman, 1972). Like the intensive care patients and those with less severe NTI, dialysis fT_3 levels were low in this condition but in contrast to those in intensive care, TSH con-

centrations were well-maintained and dialysis fT_4 values were borderline raised. Studies in a uraemic rat model show a decrease in both the activity of certain intracellular enzymes as in hypothyroidism (Lim et al., 1980) and the T_3-receptor binding in liver and kidney (Thompson et al., 1980) whereas the pituitary T_3 content is normal (Lim et al., 1983). These patients, therefore, do not have "compensated hypothyroidism" but appear to have some physiological resetting of the HPT axis. Alternatively, they may have impaired uptake of thyroid hormone by the liver and peripheral tissues. This may be an adaptive mechanism to spare essential protein as these patients are in negative nitrogen balance (Utiger, 1980).

It has recently been suggested that the high rT_3 concentrations in severe illness may exert effects on the rate of T_3 formation in the pituitary and brain cortex (St. Germain et al., 1986). Subtle differences in pituitary physiology may, therefore, occur in CRF compared to other low T_4 syndromes since serum rT_3 levels are normal in this disease.

(d) Thyroxine Replacement Therapy

In patients taking T_4, there may be a marked divergence in the clinical impression of thyroid status from that suggested by the results of biochemical tests (Fraser et al., 1986). Basal TSH assay is accepted as

the best test of inadequate replacement (Cotton et al., 1971) but its use in indicating over-treatment is controversial. Clinical signs of excessive treatment are often not apparent until total T_3 concentrations are raised and since T_3 is the most active thyroid hormone, it is argued that provided the serum total T_3 or fT_3 concentrations are maintained within the reference range, over-treatment, if it occurs, is of little consequence unless there are other contraindications e.g. ischaemic heart disease (Pearce & Himsworth, 1984; Rendell & Salmon, 1985; Fraser et al., 1986). In this study, however, many patients with total T_3 and fT_3 levels within reference limits had evidence of tissue hyperthyroidism from measurement of various serum constituents (Chapter 7). This is consistent with reports where other end-organ responses have been studied (Goolden et al., 1971; Jennings et al., 1984; Beckett et al., 1985; Coindre et al., 1986; Wilcox & Levin, 1986) but in disagreement with studies using clinical signs alone (Pearce & Himsworth, 1984; Fraser et al., 1986). A high serum fT_4 or undetectable TSH IRMA were better indicators of these subclinical changes in tissue thyroid status. Although the pituitary is more sensitive to circulating T_4 compared to other tissues, several authors have advocated maintaining the pituitary responsiveness to TRH, since mild hyperthyroidism is difficult to detect clinically (Evered et al., 1973; Wehmann et al., 1983; Davis

et al., 1984; Toft, 1985). As a result, lower daily doses of 100-125 μg are being recommended for average replacement requirements, particularly in the elderly (Davis et al., 1984; Hennessey et al., 1986). Since some patients require high fT_4 levels to normalise TSH, and fT_3 is an insensitive test of tissue hyperthyroidism, this study shows that measurement of basal TSH by sensitive assay provides a more accurate measure of thyroid status in patients receiving T_4.

(e) Special Cases

As discussed in Chapter 7, basal TSH measurements do not accurately measure thyroid status for the first 3 months after therapy for hyperthyroidism due to delayed recovery of the thyrotrophs. In addition, there are rare instances of hyperthyroidism due to a TSH secreting tumour or thyrotroph hyperplasia in which the usual negative feedback control is destroyed as in secondary hypothyroidism. In these circumstances, although the TSH measurement may be providing accurate information it conflicts with the thyroid hormone status of the patient.

A rare condition termed peripheral resistance to thyroid hormones has also been described where the high total and free thyroid hormone concentrations may be misinterpreted as hyperthyroidism. Such patients are clinically euthyroid, have normal thyrotroph responsiveness and normal basal TSH concentrations. The high thyroid hormone concentrations are necessary to compensate

for a partial defect in either tissue-uptake, peripheral T_4 conversion or thyroid hormone receptors (Degroot, 1979; Jansen et al., 1982; Wortsman et al., 1983). Patients treated with drugs e.g. iopanoic acid or amiodarone which interfere with peripheral T_4 conversion, may also have high fT_4 concentrations which contradict the clinical impression of euthyroidism and the normal fT_3 and basal TSH (IRMA) concentrations found.

In the special circumstances described, one biochemical test may be misleading but an alternative test of free thyroid hormones in patients with hypothalamic-pituitary disease or a test of end-organ response (i.e. TSH IRMA) in patients where there is peripheral resistance, will give an accurate measure of thyroid status.

8.1.3 Conclusions

From the foregoing discussion, it would appear that assessment of end-organ response by sensitive TSH measurement should provide the most accurate indicator of thyroid status in most situations. In general, measurement of fT_4 by reference techniques also correlates with the clinical impression and/or measurements of end-organ response. However, free hormone measurements by analogue assay give analytically invalid results in some patients which may reduce their clinical value substantially.

8.2 THE CLINICAL VALUE OF ANALOGUE FREE THYROID HORMONE ASSAYS AND TSH IRMA

In this section,the clinical value of analogue free thyroid hormone tests and measurement of TSH IRMA is assessed in the three main situations where requests for thyroid function tests are made.

8.2.1 Reasons for Requesting Thyroid Function Tests

The main reasons for requesting thyroid function tests are:-

(a) to confirm thyroid dysfunction when the clinical suspicion is high.

(b) to screen for thyroid dysfunction.

(c) to monitor the response to treatment.

In patients referred to a thyroid clinic, the clinician often has to distinguish hyperthyroidism, which may require destructive therapy, from euthyroidism. In this situation, the biochemical tests are required to confirm the clinical impression and provide a measure of the degree of dysfunction which will, in turn, influence patient management. Biochemical tests, used for this purpose, have to be highly specific and sensitive to avoid a misdiagnosis. This is particularly important if unnecessary thyroid ablation is to be avoided.

Unrecognised, untreated hypothyroidism is associated with a high degree of morbidity. In contrast, the inappropriate treatment of euthyroid patients with T_4 is thought to have relatively low morbidity. Any

biochemical test should, therefore, have high sensitivity, particularly as in most cases of hypothyroidism assessment and treatment will be initiated by a general practitioner rather than an endocrinologist.

The biochemical screening of elderly patients can be justified by the high prevalence (over 5%) of thyroid disease in this group (Tunbridge et al., 1977), and the fact that classical symptoms of thyroid disease are often absent or disguised by systemic illness. The yield of detecting unsuspected disease with total T_4 measurements in the elderly has been rewarding unlike the screening carried out in the general hospital population (Lancet Editorial, 1983). The latter practice places greater demands on biochemical tests to have high specificity to minimise the performance of fruitless further tests. Wilke and Eastment (1986) recently recommended the FT4I as the single best screening test but they did not evaluate the performance of sensitive TSH measurements.

Thyroid function tests are also required in the follow-up of patients treated for thyroid disease (a) to detect relapse of hyperthyroidism or the onset of hypothyroidism and (b) to monitor compliance and the adequacy of T_4 replacement therapy.

8.2.2 Confirmation of Suspected Thyroid Dysfunction

The predictive values for TSH IRMA and free thyroid hormone measurements by analogue assay were calculated in Chapter 4 for patients referred to a thyroid

clinic. The basal TSH (RIA) or response to TRH were used as the gold standards to which the new tests were compared. This allowed assessment not only of their performance in confirming the clinically obvious but also in identifying subclinical disease.

Basal TSH measurements by IRMA showed excellent correlation with the TSH (RIA) response to TRH (Chapter 4). This confirms the early work of Wide & Dahlberg (1980) and Wehmann et al. (1983) who developed TSH RIA methods with sensitivities of 0.3 mU/l, and is supported by the results from other immunometric assays (Alexander et al., 1984; Weeks et al., 1984; Paterson et al., 1985; Martino et al., 1986; Clark & Price, 1986). This test had the highest diagnostic efficiency compared to total and free thyroid hormone measurements and could be used to replace the TRH test leading to less inconvenience for patients and savings in material and staff costs.

Although free-hormone measurements identified about 50% of patients with subclinical thyroid disease, basal TSH IRMA distinguished all of these patients. This is of clinical value since these patients should be followed up as they may require treatment at a later date. Indeed, half of the patients identified with subclinical disease in this study have now been treated with T4 replacement or anti-thyroid therapy. In other studies,

fT_4 measurement has proved a good test of subclinical hypothyroidism (Faglia et al., 1982; Symons et al., 1983; Wilke & Eastment, 1986) but this was not confirmed here (Chapter 4). Similarly, measurement of fT_3, advocated by some as a better test of hyperthyroidism (Carayon et al., 1979; Franklyn et al., 1984) was no better than fT_4 in this study since most patients with T_3-thyrotoxicosis had raised fT_4 concentrations, and patients with subclinical disease more frequently had raised fT_4 values, in agreement with the findings of Weeke and Orskov (1979). Free hormone measurements are more sensitive tests of thyroid status than total hormones in this situation because they are unaffected by the small changes in TBG concentration which occur as a result of thyroid dysfunction (Burr et al., 1977).

In a proportion of patients referred to thyroid clinics, the abnormal total T_4 and T_3 results are due to high oestrogen levels and, more rarely, to the presence of abnormal serum binding proteins (Table 8.1). Analogue free hormone measurements provide a more specific test when there are large changes in TBG concentration but give spurious results in the presence of high affinity albumin or TBPA variants and anti-thyroid hormone antibodies (Chapter 5; Pearce & Byfield, 1986). The basal TSH IRMA measurement gives an accurate measure of thyroid status in all such patients (Chapter 5) and is of particular clinical value when underlying thyroid disease is present.

Table 8.1 Alterations in Thyroid Function Tests Not Due to Thyroid Dysfunction

	Analogue						
	Total T_4	Total T_3	Free T_4	Free T_3	TRH Test	Basal Sensitive TSH	Additional Reference
Age	N	N or ↓	N	N or ↓	N or ↓	N	[a]Kreutzer et al., 1986; [b]Harman et al., 1984
Early pregnancy	N or ↑	N or ↑	N	N	N or ↓	N or ↓	[b]Guillaume et al., 1985
Late pregnancy	↑	↑	N or ↓	N or ↓	N	N	"
Starvation or prolonged fasting	N	↓	N	↓	↓	↓	[a]Findlay et al., 1987
Nephrotic syndrome	↓	↓	↓	↓	N	N	Chopra et al., 1983
Chronic liver disease	N or ↓	↓	↓	↓	N	N	"
Hepatitis	N or ↑	N or ↑	N	↓	N	N	"
Chronic renal failure	↓	↓	↓	↓	N or ↓	N	"
Severe non-thyroidal illness	↓	↓	↓	↓	N or ↓	N or ↓	[b]Wehmann et al., 1985
Recovery from severe illness	N	N or ↓	N	N or ↓	N or ↑	N or ↑	"
Major depression	N or ↑	N	N or ↑	N	N or ↓	-	Extein et al., 1980
Oestrogens, tamoxifen, familial TBG excess	↑	↑	N	N	N or ↑	N	Franklyn et al., 1983
Androgens, familial TBG deficiency	↓	↓	N	N	N	N	Waud et al., 1983
Analbuminemia and albumin deficiency	N	N	↓	↓	N	N	Stockigt et al., 1983
Familial dysalbuminemic hyperthyroxinemia	↑	N	↑	N or ↑	N	N	Hennemann et al., 1982
Anti-thyroid hormone antibodies	*	*	↑	↑	N	N	Beck-Pecoz et al., 1982
Peripheral thyroid hormone resistance	↑	N or ↑	↑	N or ↑	N	-	Wortsman et al., 1983
Corticosteroids	↓	↓	N	N	↓	-	Wehmann & Nisula, 1984
Dopamine	-	-	-	-	↓	↓	[b]Wehmann et al., 1985
Dopamine antagonists	-	-	-	-	N or ↑	-	Weetman et al., 1981
Lithium	↓	↓	↓	↓	N or ↑	N or ↑	[b]Wehmann & Nisula, 1984
Phenytoin, salicylate, fenclofenac, furosemide	↓	N or ↓	↓	↓	N or ↓	-	Burger et al., 1982; Stockigt et al., 1985
Heparin (NEFA)	N or ↑	N	↓	↓	N	-	Bayer, 1983b
Iopanoic acid	↑	N or ↓	↑	N or ↓	N	-	Burger et al., 1982
Amiodarone	↑	N or ↓	↑	N or ↓	N or ↓	N or ↓	Burger et al., 1982; [a]Weirsinga et al., 1986

*Results depend on the separation system used for RIA. Sensitive TSH assays: a) Immunometric b) RIA.

Abnormally low analogue fT_4 and fT_3 values may occur in the third trimester of pregnancy and although the progressive fall in values with gestation agrees with current understanding of the physiology, this is partly due to methodological artefacts (Chapter 5). When trimester-related reference ranges are used, these measurements are of value in distinguishing hyperthyroidism from euthyroidism in pregnancy and may be of value in monitoring anti-thyroid treatment (Momotani et al., 1986). A normal basal TSH IRMA excludes thyroid dysfunction in pregnancy and this is particularly useful since the TRH test is contra-indicated. However, in a few patients (5%), there may be a physiological reduction in TSH secretion to undetectable levels (Chapter 5). If free thyroid hormone concentrations are within the appropriate reference limits for gestation, subclinical hyperthyroidism cannot be excluded by biochemical tests but this may not be of pathological significance.

It is generally accepted that free rather than total thyroid hormone measurements have greater clinical value in uncomplicated patients (Wilke 1986; Pearce & Byfield, 1986). Whether they significantly improve upon the categorisation achieved by the FT4I or T4:TBG ratio is disputed (Wilke, 1982; Braun et al., 1983; Wilke & Eastment, 1986) but they allow significant savings in

terms of time and labour. Basal TSH measurements by sensitive assay, however, have the best diagnostic efficiency in uncomplicated patients.

8.2.3 Screening for Thyroid Disease

In general, in medical admissions to hospital and patients with COAD or CRF (Chapter 6), free thyroid hormone measurements by analogue assay were of less clinical value than total hormone measurements producing several low results which reflected the concentration of albumin and the albumin-binding of certain drugs rather than thyroid status. This is in agreement with several other authors e.g. Vermaak et al.(1983). Nystrom et al. (1986a) however, recently reported few misleading results in non-hospitalized patients with NTI using analogue fT_4 RIA. Factors causing abnormal results are shown in Table 8.1 and contrasted with effects on total hormone measurements, the TRH test and basal TSH IRMA.

Measurements of fT_4 and fT_3 by non-analogue commercial methods give results of greater validity than analogue RIA in patients with NTI (Chan et al., 1983; Ordonez-Llanos et al., 1984; Lee et al., 1986; Jackson & Ekins, 1986). Basal TSH (IRMA) measurement gave the lowest number of abnormal results in keeping with the known prevalence of thyroid disease (Chapter 6). Raised concentrations were not due to lithium therapy or dopamine antagonists and the patients were not in the recovery phase from severe illness. Although not all of those

with raised TSH had anti-thyroid antibodies, a recent study by Sawin et al. (1985) concluded that demonstration of a clearly elevated TSH is a better approach to detect early thyroid failure than measurement of autoantibodies, particularly in the elderly. Although fasting and severe NTI can suppress TSH secretion, this has only resulted in normalisation of TSH levels in patients with subclinical rather than overt hypothyroidism (Borst et al., 1983; Hooper, 1976; Slag et al., 1981b). Secretion of TSH is much less affected by NTI and drugs than thyroid hormone levels (Table 8.1) and, therefore, as with the uncomplicated patient, provides the best test of primary hypothyroidism.

In geriatric patients (Chapter 6), relatively few patients had low total and free T_4 levels which was unexpected in view of their systemic illness and range of drug therapies. However, recent studies suggest that total and free T_4 concentrations remain unchanged in old age (Lindstedt et al., 1983; Harman et al., 1984), and since a significant proportion of the patients studied were fully mobile and attending a day clinic, the disease severity was probably less in this group than seen in the admissions to the general medical ward. Most geriatric patients with low thyroid hormone results had normal TSH concentrations and since TSH measurement has greater specificity as discussed above, this is the test of choice to screen for primary hypothyroidism in the elderly.

All of the thyroid function tests investigated lacked specificity for hyperthyroidism to some extent in patients with systemic illness (Table 8.1) and in the elderly (Chapter 6). Measurements of total and free thyroid hormones, however, may lack sensitivity in the presence of NTI (Engler et al., 1978; Forfar et al., 1979) and in the very old (Tibaldi et al., 1986). Undetectable basal TSH (IRMA) levels occurred more frequently than high total or free thyroid hormone concentrations and were found in 1% of medical ward patients and 4.5% of geriatric patients; thyroid hormone results were consistent with the presence of subclinical hyperthyroidism in half of these patients but thyroid dysfunction could not be firmly established. Boye & Weeke (1986) have recently shown low TSH (IRMA) concentrations with raised fT_4 levels by ultrafiltration-RIA in several patients with NTI. However, this thyrotroph suppression may be temporary as demonstrated in two geriatric patients in this study and by Davies et al. (1985) in elderly patients with atrial fibrillation. The clinical value of using basal TSH IRMA to screen for hyperthyroidism in the elderly is, therefore, less well established but if one test is to be used, it will produce a similar number of follow-up requests as total T_4 or fT_4, and provide a specific test of hypothyroidism and a sensitive test of hyperthyroidism.

8.2.4 Monitoring the Response to Therapy

Sensitive TSH measurements have no clinical value in testing for the occurrence of hypothyroidism in the early months following treatment of hyperthyroidism. Total and free T_4 measurements performed equally well for this purpose (Chapter 7). However, Martino et al. (1982) demonstrated in methimazole-treated patients that normalisation of total T_4 precedes that of fT_4 and that the fT_4 results were in keeping with the persistence of clinical hyperthyroidism. These workers have suggested that measurements of free thyroid hormones might provide a more reliable index of thyroid status in these patients.

The results of the present study of biochemical monitoring of T_4 replacement therapy (Chapter 7), show that changes in peripheral tissue markers indicative of over-treatment occur when fT_4 levels are high, basal TSH IRMA is undetectable, and when total T_4, total T_3 and fT_3 levels are often normal. Abnormalities in these markers (e.g. GST) can be reduced and levels of thyroid hormones generally maintained within reference ranges by adjusting the T_4 dosage to give normal and detectable TSH IRMA levels. Measurement of TSH can therefore indicate both over- and under-replacement and be used to optimise the replacement dose more accurately. Free or total T_4 measurement may be useful to indicate the amount of adjustment required and to check for patient compliance.

There are some circumstances where the therapeutic aim in giving T_4 is to suppress TSH secretion completely e.g. in patients with thyroid cancer or goitre due to Hashimoto's thyroiditis. Sensitive TSH measurements may provide a more accurate means for determining the dose which gives complete suppression without inducing mild hyperthyroid symptoms (Spencer et al., 1986).

8.3 NEW STRATEGIES OF THYROID FUNCTION TESTING

8.3.1 The Objectives of a Test Strategy

Ideally, the best assessment of thyroid status would involve the provision of several tests on each patient. Indeed Wilke and Eastment (1986) have suggested that the combination of total T_4, FT_4I, total T_3 and analogue fT_3 tests provides the best screening of patients when little information is available as to their possible thyroid status. However, some compromise has to be reached in terms of reagent and labour costs, and diagnostic effectiveness. The operation of strategic testing can result in a 50% cost-saving compared to unrestricted requesting of thyroid function tests (Beck, 1986). To be diagnostically effective, any proposed strategy should be applicable in the clinical situations outlined in Section 8.2.1 and aim to exclude thyroid dysfunction at an early stage to avoid the performance of unnecessary tests.

A new strategy of thyroid function testing is proposed in Figure 8.1 as a result of the clinical studies detailed in earlier chapters. Benefits of such an approach include (a) simplification of test strategy, (b) identification of relatively minor degrees of thyroid dysfunction (i.e. good sensitivity), (c) a reduction in the number of misleading abnormal results in patients with NTI (i.e. good specificity) and (d) improved biochemical control of patients taking T_4 replacement. A single sensitive TSH measurement as the initial test divides patients into those who are euthyroid (or adequately replaced with T_4) in whom no further tests should be needed, and those with overt or subclinical thyroid dysfunction (or sub-optimal T_4-replacement). Free T_4 (analogue assay) provides a slightly better second-line test than total T_4 for reasons discussed in Section 8.2. The second-line test provides biochemical evidence of the severity of the thyroid disturbance in those with an abnormal TSH level. Measurement of fT_3 or total T_3 would only be required in a small proportion of patients if TSH was undetectable and fT_4 normal, in order to exclude the few cases of T_3-thyrotoxicosis in which fT_4 was not also raised.

Analogue free hormone measurements may give falsely low results in patients with NTI who may also have undetectable TSH. However, this should be a signal to

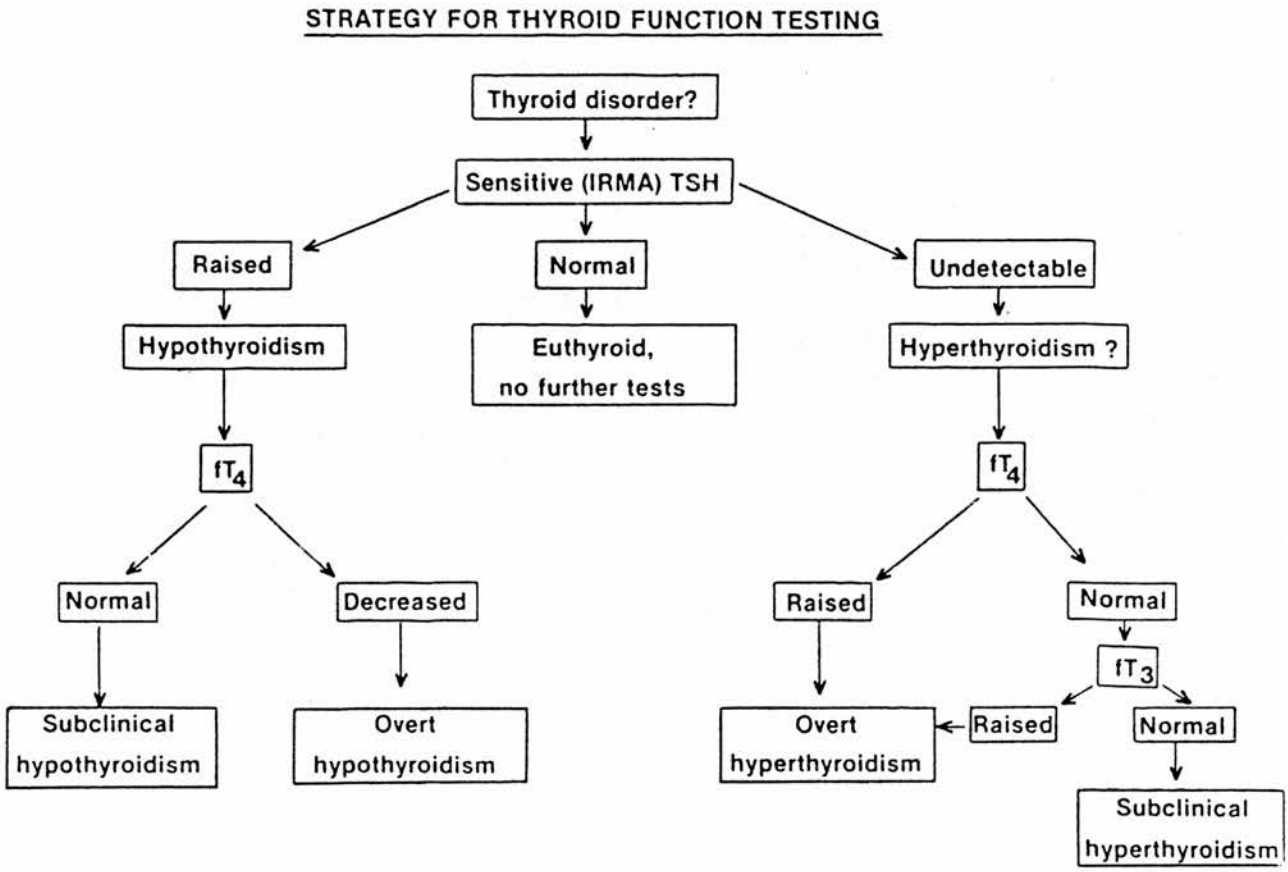


Figure 8.1 A Proposed New Strategy of Thyroid Function Testing Using Sensitive TSH Measurement as the First-line Test.

suspend, where possible, further thyroid investigations until the resolution of the systemic illness. The occurrence of false negative results using TSH as a first-line test is important but likely to occur only in the relatively rare conditions of hypopituitarism or TSH-secreting tumour. When hypopituitarism is suspected clinically or when anti-thyroid therapy has been given in the previous 3 months, basal TSH measurement is inappropriate and fT_4 measurement should be performed. A TRH test may also be useful in suspected hypopituitarism.

The potential for non-thyroidal causes of an undetectable TSH (Table 8.1) has led to some reservations as to the use of TSH as the first-line test, particularly in hospital in-patients (Kirkegaard et al., 1985; Nystrom et al., 1986b). However, Semple et al. (1985), using the Boots-Celltech TSH IRMA for their routine workload (endocrine clinic and general hospital), found only 9 out of 3629 patients (0.25%) with values <0.1 mU/l not explicable by hyperthyroidism, T_4-therapy, treatment of hyperthyroidism or remission from Graves' disease. Experience in this laboratory of operating a strategy of Amerwell TSH IRMA as the first-line test, followed by a total T_4 if TSH is abnormal for 1916 routine requests, has yielded similar results: TSH was low (<0.3 mU/l) and total T_4 normal in 27 (1.4%) patients and, of these, 13 (<0.7%) were undetectable and could not be explained by T_4 replacement or clinically apparent thyroid disorder.

The outcome of using a sensitive TSH measurement as a first-line test is that it will identify a group of previously unrecognised patients with subclinical disease. The question must be asked as to whether this is an advantage. There is little dispute that the detection and review of patients with subclinical hypothyroidism is important since the natural history is progression to overt disease and there is some evidence that there are changes in end-organ function (Tunbridge et al., 1977; Cooper et al., 1984). Although the outcome of long-term replacement in asymptomatic patients has not been assessed, there may be some benefit in patients with abnormal myocardial contractility or symptoms consistent with mild hypothyroidism (Cooper et al., 1984). There is less of a consensus in the view that subclinical hyperthyroidism inevitably progresses to overt disease (Studer et al., 1985),but studies in patients with solitary non-toxic autonomously-functioning thyroid nodules indicate that these progress to toxic adenomata (Hamburger , 1980; Lerro et al., 1985) and there may be slow progression to hyperthyroidism in patients with multinodular goitre (Studer et al., 1985). Treatment at an early stage in such patients may, therefore, be considered beneficial. Patients who are clinically euthyroid, with normal thyroid scans and who are in sinus rhythm would not be candidates for treatment but should be

reviewed to exclude effects of NTI and to detect any progression to overt thyroid disease (Semple et al., 1985). The new strategy (Figure 8.1) would therefore increase the need for follow-up of patients with minor degrees of thyroid disease.

It has been calculated that the proposed strategy (Figure 8.1) could halve the number of biochemical tests performed in a thyroid clinic with additional savings due to not performing TRH tests (Caldwell et al., 1985; Martino et al., 1986). However, for workloads derived from populations with a low prevalence of thyroid disease, such an analysis will not apply, particularly when in-house total T_4 assays are used as screening tests (Beck, 1986). Any analysis of the comparative cost of one strategy compared with another has to take into account the population served, the cost of running in-house assays compared to commercial kits, staffing, equipment and various local factors.

Developments in dual-isotope immunoassay provide further scope for the simplification and improved efficiency of thyroid function testing. Single-tube, dual-isotope methods for simultaneous measurement of TSH by IRMA and fT_4 by analogue RIA will soon supercede the dual RIA evaluated in this thesis (Chapter 4). This proved very effective in assessing the thyroid status of new thyroid patients, pregnant women and patients with co-

existent illness. Preliminary results in patients from a thyroid clinic with such a dual TSH IRMA/fT_4 RIA are shown in Figure 8.2. In contrast to the performance of the dual RIA kit (Figure 4.12), TRH testing might not be necessary to distinguish euthyroidism from subclinical hyperthyroidism. The performance of this assay as a first-line test would lead to 9 possible combinations of results (Figure 8.3); four of these combinations clearly identify the thyroid status and no further tests would be required: (a) overt hyperthyroidism (e) euthyroidism (f) subclinical hypothyroidism (i) overt hypothyroidism. Patients with low TSH IRMA but normal fT_4 (Figure 8.3(d)) would require measurement of total or free T_3 to identify patients with T_3-hyperthyroidism. Patients with low fT_4 and either low or normal TSH IRMA (Figure 8.3(g & h)) might require a TRH test if hypopituitarism is suspected clinically. The main disadvantage of the combined test would be in the detection of patients with euthyroid (or hypothyroid) hyperthyroxinemia (Figure 8.3(b & c)) in whom further tests might be required if the clinical findings were equivocal. A TSH-secreting tumour would give a similar combination of test results. The advantages of this approach would be:-

1. A unified strategy (even suitable for monitoring patients after treatment of hyperthyroidism).

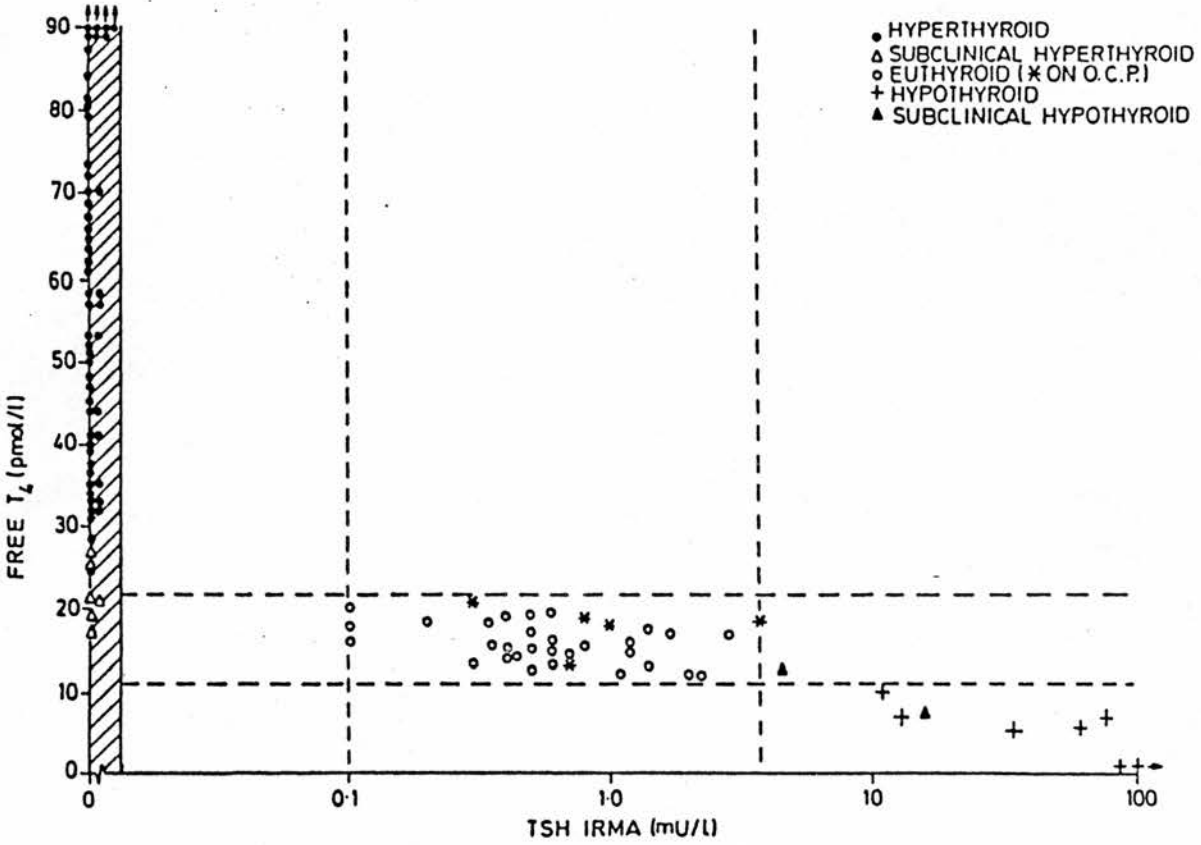


Figure 8.2 Preliminary Results for the Becton Dickinson Dual-Isotope TSH IRMA/Free T4 RIA in New Patients from a Thyroid Clinic.

Results in the hatched area had counts in the TSH IRMA less than the zero standard. Dashed lines indicate the absolute range of values for TSH and the 95% confidence limits for fT_4.

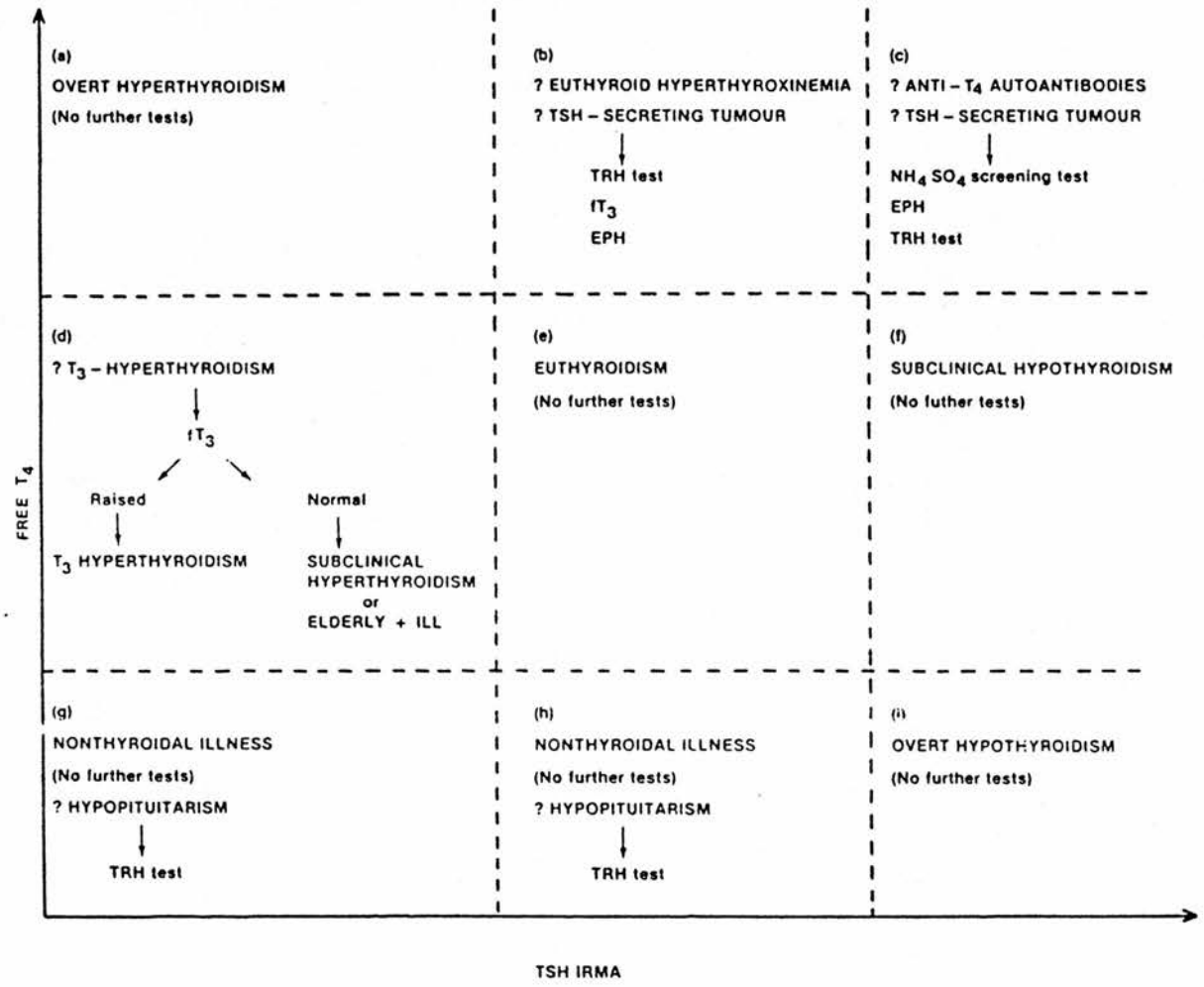


Figure 8.3 The Possible Combinations of Results Using a Simultaneous TSH IRMA/Free T4 RIA and the Requirement for Further Tests.

2. Earlier indication of the degree of thyroid hormone excess in untreated hyperthyroidism and in patients taking T_4.
3. Improved detection of hypothalamic-pituitary dysfunction.
4. Easier laboratory processing of patient samples and data.

Since such a dual assay might cost the equivalent of either a commercial sensitive TSH measurement or analogue fT_4 assay, such a strategy is likely to be most beneficial to the patient, clinician and the laboratory.

8.4 CONCLUSIONS

In patients presenting to a thyroid clinic, measurements of free thyroid hormones by analogue-RIA provide a better test of thyroid status than total hormone measurements. This is not the case for hospital inpatients primarily due to artefacts arising from the methodology. Basal TSH measurement by immunometric assay, however, provides a more sensitive and specific test of thyroid dysfunction and is therefore better suited as a screening test and as a confirmatory test when the clinical suspicion is high. In hypothyroid patients treated with T_4, basal TSH measurement may now provide the best biochemical indicator of both over- and under-replacement. The use of basal TSH as a first-line test has major advantages over using a measure of total or free T_4 alone in a) decreasing the number of unnecessary tests and b) identifying subclinical disease. In addition to having a major impact on the design of strategies for thyroid function testing, the new generation of sensitive TSH assays will also provide a better tool in studies of hypothalamic-pituitary-thyroid physiology.

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APPENDIX I

List of Abbreviations

ACE	Angiotensin-converting enzyme
ALT	Alanine aminotransferase
ANS	Anilino-naphthalene sulphonic acid
CK	Creatine kinase
COAD	Chronic obstructive airways disease
CV	Coefficient variation
DARS	Donkey anti-rabbit serum
DAS	Donkey anti-sheep serum
EDTA	Ethylenediaminetetra-acetic acid
FD	Final dilution
FDH	Familial dysalbuminemic hyperthyroxinemia
fT_3	Free triiodothyronine
fT_4	Free thyroxine
FT_4I	Free thyroxine index
GGT	Gamma-glutamyltransferase
GST	Glutathione S-transferase (B_1B_1)
HEPES	N-2-hydroxyethulpiperazine-N'-2-ethane-sulphonic acid
HPT	Hypothalamic-pituitary-thyroid
IRMA	Immunoradiometric assay
IRP	International reference preparation
NEFA	Non-esterified fatty acids
NSB	Non-specific binding
NSS	Normal sheep serum
NTI	Non-thyroidal illness
RAC	Repeat analysis control
RIA	Radioimmunoassay
rT_3	Reverse triiodothyronine
SAPU	Scottish Antibody Production Unit
SHBG	Sex hormone binding globulin
SMAC	Sequential Multiple Analyser plus Computer
T_3	Triiodothyronine
T_4	Thyroxine
TBG	Thyroxine binding globulin
TBPA	Thyroxine binding prealbumin
TRH	Thyrotrophin-releasing hormone
TSH	Thyrotrophin (Thyroid stimulating hormone)

APPENDIX II

Published Papers

The following papers were published as a result of work presented in this thesis (reproduced with permission of my co-authors and the publishers).

CLIN. CHEM. 31/11, 1888–1892 (1985)

Accuracy and Precision of Five Analog Radioimmunoassays for Free Thyroxin Compared

S. M. Gow,[1] H. A. Kellett,[2] A. D. Toft,[2] and G. J. Beckett[1]

We compared the precision of free thyroxin (FT_4) measurements by kits involving analog RIA and the use of antibody-coated tubes (Becton Dickinson and Coat-A-Count), magnetic separation (Amerlex Magnetic and Corning Magic), or centrifugation of antibodies linked to solid beads (Amerlex). Results of kits with magnetic separation were the most reproducible. Amerlex, Amerlex Magnetic, and Becton Dickinson kits gave values comparing best with those obtained by direct equilibrium dialysis. Coat-A-Count and Corning Magic results differed significantly from dialysis values, both for patients' samples and kit standards. The kits had equal diagnostic efficiency in patients with suspected thyroid disease. On measurement of FT_4 some patients were reclassified from "subclinical thyroid disease" to "overt disease." Most patients with triiodothyronine thyrotoxicosis had increased FT_4. Several kit values were low for pregnant women and patients with nonthyroidal illness but the Amerlex and Amerlex Magnetic assays had fewer low results. The Amerlex Magnetic FT_4 assay gave the best precision, agreement with the reference method, and diagnostic efficiency.

Additional Keyphrases: *thyroid status · variation, source of · pregnancy · "kit" methods · diagnostic efficiency*

Estimation of free thyroxin (FT_4) in serum is generally considered to provide a more nearly accurate diagnostic test of thyroid dysfunction than is measurement of total thyroxin (T_4) in serum or determination of the free thyroxin index (FTI), particularly in conditions where there are changes in the concentrations of thyroid hormone-binding proteins such as in pregnancy (*1–3*).[3] It is also currently held that only the free fraction of thyroid hormones can gain access to cells and thus exert metabolic effects (*4, 5*). The development by Amersham International of the one-step RIA involving a radiolabeled analog of thyroxin (*6*) has resulted in FT_4 assays that are more precise and robust than earlier commercial methods involving microencapsulated antibodies and two-step kinetic rate analysis (*7*). These advantages have led many companies to adopt this methodology, and several FT_4 RIA kits now marketed are based on the use of different T_4 analogs and separation systems.

Many studies evaluating individual FT_4 kits have compared them with the FTI and other thyroid-function tests rather than with a reference method for FT_4. Equilibrium dialysis of serum followed by RIA of T_4 in the dialysate is the reference method for FT_4 but is unsuitable for routine use (*5*). Here we report our assessment of five different FT_4 analog assays having various separation systems. We have compared these assays for (*a*) precision, (*b*) accuracy as compared with a reference dialysis method, and (*c*) diagnostic efficiency in patients with suspected thyroid disease, in pregnant women, and in patients with nonthyroidal illness.

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[3] Nonstandard abbreviations: FT_4, free thyroxin; FTI, free thyroxin index; T_4, total thyroxin; $FT_4(D)$, FT_4 as measured by RIA after dialysis to equilibrium; TSH, thyrotropin (thyroid-stimulating hormone); TRF, thyroliberin (thyrotropin-releasing factor); and T_3, triiodothyronine.

Received May 8, 1985; accepted August 1, 1985.

Materials and Methods

FT_4 by Equilibrium Dialysis

Samples were diluted 20-fold with pH 7.4 buffer containing, per liter, 10 mmol of 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES; Sigma Chemical Co.), 106 mmol of NaCl, and 1 mmol of NaN_3, then dialyzed against an equal volume of this buffer at 37 °C in a "Dianorm" equilibrium dialyzer (Diachema AG, Zurich, Switzerland) for at least 18 h. The T_4 in the dialysates was then measured in duplicate by RIA [$FT_4(D)$] with a pre-precipitated double-antibody system (*8*). Between-run precision was assessed by dialysis and RIA of three serum pools in 15 assays. The mean precision (CV) was 11% for FT_4 values in the range 7 to 27 pmol/L.

FT_4 by Analog RIA

We studied FT_4 kits from the following manufacturers: Amerlex and Amerlex Magnetic (Amerlex-M) (Amersham International, Amersham, Bucks., U.K.); Becton Dickinson (Becton Dickinson UK Ltd., Cowley, U.K.); Coat-A-Count (Diagnostic Products (UK) Ltd., Wallingford, U.K.); and Corning Magic (Corning Medical and Scientific, Halstead, U.K.). The Amerlex-M kit, which contains the same analog and standards as the Amerlex kit, became available after the start of this study. Table 1 summarizes the assay protocols. All assays were performed by one operator, using an automatic diluter and dispensing sample with tracer in one step within 10 min to minimize effects of drift, particularly in the coated-tube assays.

Subjects

Endocrine clinic patients. Samples were collected from 200 consecutive patients referred to an endocrine clinic. These patients were categorized on the basis of clinical examination by one consultant, and results of measurements of serum T_4, total triiodothyronine (T_3), basal thyrotropin (TSH), and the TSH response 20 min after intrave-

Table 1. Comparison of FT_4 Analog RIAs

FT_4	Amerlex	Amerlex Magnetic	Becton-Dickinson	Coat-A-Count	Corning Magic
Sample vol, μL	100	100	50	50	50
Pipetting steps	2	2	1	1	2
Incubation at 37 °C, h	1	1	1.5	1	1
Wash step	No	No	Yes	No	No
Sepn. method	Centrifn.	Magnetic	Coated tube	Coated tube	Magnetic

nous administration of 200 μg of TRF (TRF stimulation test).

The results for 63 patients classified clinically and biochemically as euthyroid were normally distributed. We used them to derive reference intervals (mean ± 2SD) for each FT_4 method. The 110 hyperthyroid patients had increased concentrations of total thyroid hormones and a TSH response of <1 milli-int. unit/L in the TRF test. Five of these patients had normal values for T_4 but increased T_3; they were described as T_3-thyrotoxic. Nine patients had a flat TRF test response but normal concentrations of T_4 and T_3; these were classified as having subclinical hyperthyroidism. Eight patients with low T_4 and increased TSH were classified as having overt primary hypothyroidism. Ten patients had normal T_4 values but an above-normal TSH concentration and were categorized as having subclinical hypothyroidism.

Pregnant women. Samples were collected from 93 women attending an antenatal clinic at various stages of pregnancy.

Patients with nonthyroidal illness. Two groups of patients were studied: (*a*) 39 patients from a general medical ward, including patients with cardiovascular, gastrointestinal, liver, or respiratory disease, stroke, or diabetes mellitus, and (*b*) 36 patients with chronic renal failure, who were receiving dialysis treatment. All of these patients had normal concentrations of TSH but 28% of the T_4 and T_3 results were low in group *a* and 67% of T_4 and 36% of T_3 results were low in group *b*.

All sera were stored at −20 °C until analysis. We used Student's paired and unpaired *t*-tests and the Wilcoxon matched-pairs test for statistical analysis.

Results

Precision: Figure 1 illustrates the mean within-assay precision profiles for 15 assays by each method. These profiles were derived from analysis of the duplicate measurements for samples (*9*). The relatively poor precision of the dialysis method represents the combination of imprecision originating from the dialysis and RIA steps. The Amerlex and Amerlex-M assays had the lowest profiles compared with the coated-tube assays. The Corning Magic kit was less precise at low values. For practical purposes the precision attained with all of the kits was adequate between 3 and 100 pmol/L.

We assessed the between-assay precision for the analog FT_4 methods, using two control sera (RIATRAC II and III, Becton Dickinson) and a low-concentration serum pool (Table 2). In addition, a patient's sample from the previous run was re-analyzed in the next assay (repeat-analysis control) and, for values within the range 5 to 50 pmol/L, the precision (CV) calculated from the two results on each repeat-analysis control from 10 assays was: Amerlex 9%, Amerlex-M 5%, Becton Dickinson 9%, Coat-A-Count 7%, and Corning Magic 6%.

Each test was performed in duplicate, so we compared the average number of tests per run where there was less good agreement between replicates (duplicate error). A duplicate error was indicated if the variance ratio of the replicate counts to the mean counts exceeded the arbitrary value of 20 (*9*). For each kit the mean percentage of duplicate errors per run was: Amerlex 27%, Amerlex-M 14%, Becton Dickinson 18%, Coat-A-Count 24%, Corning Magic 13%. This represents the number of tests where the replicate values required further scrutiny to determine if re-analysis was necessary.

Drift analysis: One sample in each of 10 assays was analyzed at the beginning and the end of batches of 50 samples, each run in duplicate. There was no significant drift in the Amerlex, Amerlex-M, and Corning Magic assays, but both coated-tube assays showed a positive drift of 7 to 8% ($p < 0.05$).

Accuracy: The standards provided with each kit were measured by the equilibrium dialysis method. Regression analysis showed that the supplier-assigned standard values for Amerlex (or Amerlex-M) and Becton Dickinson fell on the line of identity with values measured by dialysis. The slopes of the regression lines for Coat-A-Count and Corning Magic standards compared to FT_4(D) differed significantly

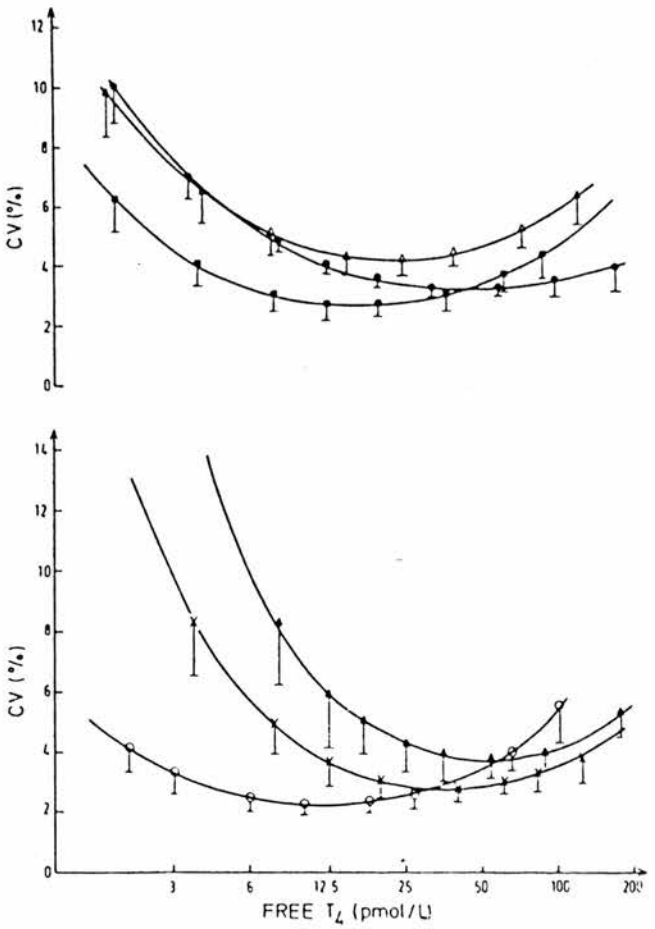

Fig. 1. Mean within-run CVs for 15 assays of FT_4 by each method
Reference intervals were: 11–23 (△, Coat A Count); 8–17 (●, Becton Dickinson, and ▲, equilibrium dialysis); 10–22 (■, Amerlex Magnetic); 10–21 (○, Amerlex); 17–30 (×, Corning) pmol/L. *Bars* indicate 1 SD

Table 2. Between-Assay Precision of the FT_4 Kits

	Amerlex	Amerlex Magnetic	Becton-Dickinson	Coat-A-Count	Corning Magic
Low-concn. pool					
n	13	16	15	10	19
Mean, pmol/L	7	7	7	8	13
SD, pmol/L	0.3	0.3	0.6	0.6	0.8
CV, %	5	5	9	7	6
RIATRAC II					
n	17	19	15	10	24
Mean, pmol/L	12	13	9	11	19
SD, pmol/L	0.6	0.6	0.8	0.8	0.9
CV, %	5	5	9	7	5
RIATRAC III					
n	15	16	16	10	24
Mean, pmol/L	35	31	21	28	38
SD, pmol/L	1.4	2.2	2.1	2.0	1.9
CV, %	4	7	10	7	5

RIATRAC II and III are commercial control sera.

from 1.0 ($p < 0.001$): FT_4 (Coat A Count) = 0.82 × FT_4(D) + 1.8 and FT_4 (Corning Magic) = 0.79 × FT_4(D) + 2.6.

Analog FT_4 values compared to FT_4(D): Figure 2 summarizes the regression analyses comparing FT_4 values obtained with four of the kits with those by FT_4(D) for 150 endocrine clinic patients. Correlation coefficients exceeding 0.94 were obtained for all of the FT_4 kits. The equations for the regression lines were: Amerlex = [1.04 × FT_4(D)] + 1.3, S_{yx} = 6.9; Becton Dickinson = [0.97 × FT_4(D)] − 0.5, S_{yx} = 6.2; Coat-A-Count = [0.84* × FT_4(D)] + 5.8*, S_{yx} = 7.1; Corning Magic = [1.00 × FT_4(D)] + 10.0*, S_{yx} = 6.2 (*significant differences, $p \leq 0.001$, from the 45 ° regression line). Amerlex and Becton Dickinson FT_4 values did not differ significantly from dialysis values but Coat-A-Count and Corning Magic values at low doses fell below the regression line drawn, indicating that this relationship was not rectilinear. For these patients, Amerlex-M values did not differ significantly from Amerlex FT_4 or FT_4(D) values: Amerlex-M = [0.97 × FT_4(D)] + 2.3, S_{yx} = 5.7.

Diagnostic efficiency in patient samples: Figure 3 shows data on the endocrine clinic patients as obtained with four of the FT_4 kits. With the Amerlex-M kit the patients were categorized in exactly the same way as with the Amerlex kit and therefore these data are not shown. The reference intervals (in pmol/L) derived from 62 euthyroid patients were: Amerlex 10–21; Amerlex-M 10–22; Becton Dickinson 8–17; Coat A Count 11–23; Corning Magic 17–30. The reference interval for FT_4(D) was 8–17.

Two euthyroid women were taking an estrogen-containing oral contraceptive, and both had increased T_4 and T_3 but normal FT_4 by all methods except the Coat-A-Count kit. One euthyroid man had a normal value for T_3, the TRF stimulation test, and FT_4(D), but above-normal values for T_4 and FT_4 with all the kits. His results were excluded when we calculated the reference intervals. On electrophoresis of his serum, this patient was shown to have increased T_4 binding to albumin.

Two patients with overt hyperthyroidism were misclassified with all of the FT_4 kits. One patient had a normal value for T_4, but increased T_3 and free T_3, and was therefore T_3 thyrotoxic. The other misclassified patient had borderline increases in total T_4 and T_3. Four of the five patients categorized as T_3 thyrotoxic had clearly above-normal FT_4 concentrations as measured by all of the kits.

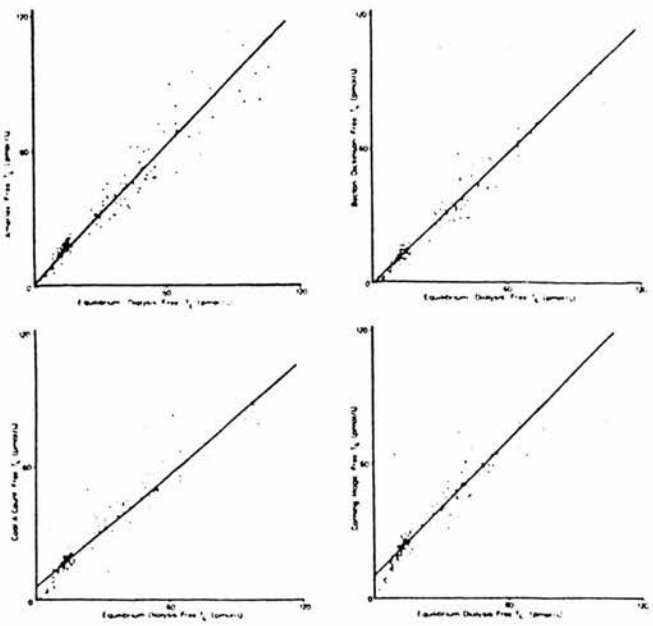

Fig. 2. FT_4 values by analog RIA and equilibrium dialysis compared for serum from endocrine-clinic patients

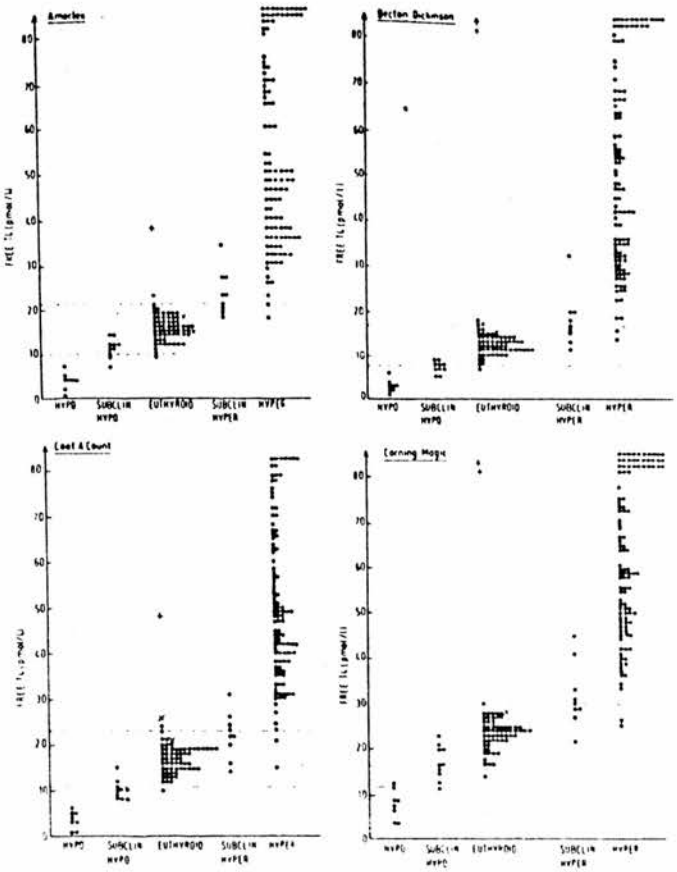

Fig. 3. Serum FT_4 as measured with four analog kits in patients classified as euthyroid, hypothyroid, or hyperthyroid, or as having subclinical thyroid disease

Two patients were taking oral contraceptives (×); one patient (+) with high FT_4 had an abnormal high-affinity albumin in the serum

In the "subclinical" groups a proportion of FT_4 results were outside of the reference intervals (Figure 3). Of the 19 patients in these groups, FT_4 results were abnormal in seven to 10, depending on the kit.

In the pregnant group, mean FT_4 values decreased significantly ($p < 0.001$) between the first and the third trimesters, by 24 to 27% as measured by all five kits. Table 3 shows the number of values falling below the reference interval for each kit.

Many of our patients with nonthyroidal illness and many of those with chronic renal failure had low FT_4 values as measured with the kits (Table 3).

Discussion

In the new generation of commercial FT_4 radioimmunoassays, T_4-analog radiolabels are used that, it is claimed, bind

Table 3. Number (and %) of Low FT_4 Values for Pregnant Women and for Patients with Nonthyroidal Illness

	Pregnancy (n = 93)	Nonthyroidal illness (n = 39)	Chronic renal failure (n = 36)
Amerlex	5 (5)	4 (10)	24 (67)
Amerlex Magnetic	4 (4)	4 (10)	24 (67)
Becton-Dickinson	4 (4)	7 (18)	30 (83)
Coat-A-Count	13 (14)	9 (23)	26 (72)
Corning Magic	11 (12)	7 (18)	29 (81)

to the assay antibody but do not bind significantly to the endogenous binding proteins in serum. The label thus only equilibrates with the free T_4 pool. These FT_4 assays are all relatively easy to perform and require short incubation times. Different manufacturers have adopted different analogs, separation systems, and methods of standardization. As we have demonstrated here, this has resulted in differences in accuracy and precision.

The precision profiles (Figure 1) showed that all the kits we studied are adequately precise over a wide range of values, the Amerlex and Amerlex-M kits having the best profiles. These kits, and the Corning Magic FT_4 assay, had better between-run precision than the coated-tube kits. In part, the imprecision found with the latter kits could be attributed to the drift effect seen in these coated-tube assays. This drift problem could be alleviated by pipetting all the samples first, followed by tracer, but this doubles the set-up time and introduces errors arising from pipetting a small sample volume. The kits in which magnetic separation is used showed fewer substantial differences between duplicates than did the other separation systems. The beads used in the Amerlex separation step appear not to form a compact pellet after centrifugation, which leads to loss of the antibody at the decanting stage and thus to imprecision and to an unacceptably high number of samples requiring reanalysis. The lower number of disagreements between duplicates with the Becton Dickinson kit as compared with the Coat-A-Count kit may be due to the wash step used in the former assay.

Analysis of the kit FT_4 standards by equilibrium dialysis showed that results for only the Amerlex (or Amerlex-M) and Becton Dickinson standards were in agreement by both kit and equilibrium dialysis, the reference method. Further confirmation of poor accuracy with the Corning Magic and Coat-A-Count methods was shown in patients' samples, where a comparison of these methods with equilibrium dialysis showed a poor fit of the data to the regression line (Figure 2). In general, the Coat-A-Count kit gave lower values than those by dialysis at high FT_4 concentrations, consistent with the results for standards. The Corning Magic kit, however, gave higher values throughout than dialysis, resulting in the higher reference range for this kit. This is not consistent with the negative bias of the kit standard values as compared with those measured by dialysis, and it suggests that some other feature of this kit such as the analog or the matrix for the standards is responsible for the difference in accuracy for patients' samples.

Despite these differences, all the FT_4 kits performed equally well in categorizing patients with thyroid disease. All but one kit correctly classified the two euthyroid women who had increased T_4 as a result of using an oral contraceptive. However, the inadequate sample size does not permit any conclusions to be drawn on the various kits' performance in this situation. Three patients were misclassified by all the kits. One patient had T_3 thyrotoxicosis and another had marginally increased T_4 and T_3 with a normal value for FT_4 by the kits and by dialysis. One euthyroid patient had an abnormal high-affinity albumin in his serum; this interfered positively with results by all the FT_4 kits, but this patient had a normal value for FT_4 as measured by equilibrium dialysis. This condition of familial dysalbuminemic hyperthyroxinemia has been well described (*10, 11*) and our data show that it affects the kit results to different extents because of the different analogs used. This abnormality is rare but, taken together with the incidence in the population of other abnormal binding proteins (e.g., anti-T_4 antibodies), which similarly interfere with analog RIA (*11, 12*), it must be recognized as a problem when these kit assays are being used. TSH assays, dialysis FT_4 measurement, and electrophoretic studies can be used to investigate further a patient with an unexpectedly high value for analog-assay FT_4 (*12*).

Of the five patients classified initially as being T_3 thyrotoxic, with normal T_4 but above-normal values for T_3, four had above-normal FT_4. Therefore, replacement of T_4 assay by FT_4 assay will apparently decrease the proportion of patients previously described as being T_3 thyrotoxic.

All of the FT_4 kits resolved the borderline cases with subclinical thyroid disease more clearly than did T_4 and T_3 measurement. Many patients had FT_4 values more consistent with overt thyroid disease. This has therapeutic implications, because some patients who were classified from T_4 assay as having "subclinical" disorder, and therefore were simply followed up, would be classified as having overt thyroid disease on the basis of the FT_4 measurement and be candidates for treatment.

For pregnant women, all the FT_4 kits showed results that decreased significantly as gestation progressed. The Coat-A-Count and Corning Magic kits gave a greater proportion of abnormally low results than did the other kits (Table 3). In practice, trimester-related reference ranges for FT_4 must be used in pregnancy if the test is to be correctly interpreted.

Low FT_4 values were found with these analog kits for several patients from a general medical ward, all of them clinically euthyroid with normal basal values for serum TSH. The occurrence of low values for T_4 in patients with nonthyroidal illness is a well-recognized diagnostic problem (*13, 14*). Contributing factors may include decreased binding capacity (or affinity) of serum protein for T_4, owing (e.g.) to proteinuria, liver disease, or drugs binding to the binding proteins. In theory, FT_4 assays should not be as greatly affected by these changes and thus should more accurately reflect thyroid status in such patients. In practice, FT_4 as measured by analog RIA is affected by changes in albumin concentration and binding capacity (*15*), but our study shows that FT_4 is still superior to T_4 measurement in these patients. The percentage of low results varied (Table 3) from 10% (Amerlex/Amerlex-M) to 23% (Coat-A-Count). In the patients with chronic renal failure, however, there was an equal or greater proportion of low FT_4 kit values as compared with T_4. Only two of our patients with chronic renal failure had low albumin concentrations and low FT_4 values have been confirmed by other dialysis methods in this patient group (*14*). Because TSH secretion is not increased, it has been argued that the thyroid axis may be "down regulated" physiologically in some patients with nonthyroidal illness, to conserve body protein and decrease catabolism (*16*). Low concentrations of T_3 in tissue, accompanied by decreased activities of thyroid hormone-dependent enzymes, have been demonstrated in uremic rats (*17*). In other studies an increase in the dialyzable fraction of T_4 has been demonstrated and attributed to the presence of inhibitors of protein binding in the sera of some patients (*18, 19*). It is becoming apparent that FT_4 may be altered in nonthyroidal illness, owing not only to method differences but also to a true physiological change, particularly in severe disease. Currently there is no clear evidence that thyroxin-replacement therapy in such patients is beneficial. If thyroid assessment is required in such patients, it therefore is advisable to use the FT_4 kit that gives the lowest number of abnormal results, and in this study the Amerlex/Amerlex-M assays fulfilled this criterion. Measurement of TSH would distinguish the low FT_4 of hypothyroidism from that of a possible physiological adaptation in patients with nonthyroidal illness.

We conclude that although all of these kits must be used with caution in patients with nonthyroidal illness, they performed equally well in classifying patients attending a thyroid clinic. However, better precision and accuracy were obtained with the Amerlex Magnetic FT_4 kit, which we consider to be the method of choice.

We thank the above-mentioned manufacturers for supplying us, gratis, with kits used in this study.

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SimulTRAC™ Simultaneous Radioimmunoassay of Thyrotropin and Free Thyroxin Evaluated

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We assessed the analytical and diagnostic performance of a dual-isotope RIA for thyrotropin (TSH) and free thyroxin (FT4) in serum. The mean interassay CVs for these analytes were 7.9% and 5.0%, respectively. The mean minimum detection limit for TSH was 0.25 milli-int. unit/L, the mean analytical recovery 110%. There was good agreement with values for TSH and FT4 measured by alternative RIA procedures. Euthyroid patients were well distinguished from those with overt thyroid disease, although there was a small overlap in the case of TSH. Combining the two results better discriminated these categories and identified those patients with subclinical thyroid disease who had abnormal TSH concentrations but FT4 concentrations within reference limits. Euthyroid women taking estrogen-containing oral contraceptives had normal results for TSH and FT4, as did most pregnant women studied. During the third trimester of pregnancy, TSH concentrations of women with low FT4 concentrations were within reference limits. Similarly, euthyroidism was confirmed in patients with low FT4 due to nonthyroidal illness by the simultaneous finding of a normal TSH concentration.

Additional Keyphrases: *thyroxin analog method · thyroid status reference interval · pregnancy*

Measurement of thyrotropin (TSH, thyroid-stimulating hormone) in serum is widely considered the most sensitive biochemical test of primary hypothyroidism.[3] In mild or subclinical hypothyroidism, total thyroxin (T4) concentrations in serum are within normal limits, indicating that an increase in TSH secretion is the first measurable change to occur when thyroid secretion declines (*1, 2*). In hyperthyroidism, the concentration of thyroxin-binding globulin (TBG) in serum decreases (*3*), making measurement of free thyroid hormone concentrations more appropriate than assays for protein-bound (total) thyroid hormones. Measurement of free thyroxin (FT4) appears to identify hyperthyroid patients almost as well as measurement of free triiodothyronine (FT3), with the advantage of greater diagnostic value than FT3 in patients with hypothyroidism (*4–6*). The "SimulTRAC™ free T4/TSH RIA," developed by Becton Dickinson, is intended to combine the diagnostic strengths of these individual measurements with the convenience of a single-tube assay. In this dual-isotope RIA, the radioactivity of the ^{57}Co-labeled thyroxin analog and ^{125}I-labeled TSH tracers is measured in a gamma counter capable of distinguishing the different scintillation energies of the two radioisotopes. The thyroxin analog is claimed not to bind significantly to the natural binding proteins in serum but binds like thyroxin to the assay antibody.

We have compared the diagnostic efficiency of this assay with our current strategy for assessing the thyroid function of patients attending an endocrine clinic. This current strategy consists of clinical assessment; measurement of total T4, total triiodothyronine (T3), and TSH in serum; and measurement of the TSH response to thyroliberin (thyrotropin-releasing factor) in cases of suspected hyperthyroidism. Because of the current controversy regarding the validity and diagnostic utility of one-step analog assays for FT4, particularly in situations of altered concentrations and affinities of hormone binding proteins (*7–9*), we also studied pregnant women and patients with low T4 concentrations related to nonthyroidal illness.

Materials and Methods

Assays

"SimulTRAC" Free T4 [^{57}Co]/TSH [^{125}I] RIA kits (five different lots) were supplied by Becton Dickinson Immunodiagnostics, Cowley, Oxford, U.K. The dual serum standards contain TSH (0 to 45 milli-int. units/L), calibrated against the WHO 1st International Reference Preparation (IRP 68/38), and FT4 (0 to 125 pmol/L), calibrated by equilibrium dialysis. In this study we used the 4-h high-sensitivity incubation option; this omits the top standard supplied, making 15 milli-int. units/L and 70 pmol/L the highest standards assayed for TSH and FT4, respectively.

We incubated 200 μL of sample with 100 μL of rabbit dual antibody for 2.5 h at 37 °C, then for 1.5 h more after adding 100 μL of dual tracer. Free ligand was separated from bound by adding goat anti-rabbit antiserum and a precipitation accelerator, polyethylene glycol, and centrifuging. Radioactivity from the two radioisotopes was counted simultaneously in a gamma counter (LKB Wallac, Wallac Oy, Turku, Finland) with <2% spill-over of counts between channels. We interpolated the results by using a four-parameter logistic curve fit (WHO immunoassay package) and an Apple microcomputer. High TSH samples (>15 milli-int. units/L) were re-assayed after fivefold dilution in the zero concentration standard.

All sera were stored at −20 °C until assay. We used Student's unpaired *t*-test and the Mann–Whitney test for unpaired data in statistical analyses.

Subjects

Endocrine-clinic patients. We collected serum from 116 consecutive new patients referred to an endocrine clinic with suspected thyroid disease or for treatment of hyperthyroidism. All 56 patients with overt hyperthyroidism had above-normal concentrations of T4 and (or) T3 and an increase in TSH of less than 1 milli-int. unit/L 20 min after the intravenous injection of 200 μg of thyroliberin. Eight patients had a flat response to this test but normal concen-

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[3] Nonstandard abbreviations: TSH, thyrotropin; T4, total thyroxin; FT4, free thyroxin; T3, total triiodothyronine; FT3, free triiodothyronine; TBG, thyroxin-binding globulin; IRP, International (WHO) Reference Preparation.

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trations of T4 and T3; they were classified as having subclinical hyperthyroidism. Forty patients with normal responses of TSH to thyroliberin were classified clinically and biochemically as euthyroid, including seven women who were taking an estrogen-containing oral contraceptive and who had increased concentrations of total thyroid hormones. Twelve patients had an increased concentration of TSH, which was associated with a low T4 concentration in nine patients (overt hypothyroidism) and a normal T4 value in three (subclinical hypothyroidism).

Pregnant women. Samples were collected from 26 women at various stages of pregnancy who were attending an antenatal clinic.

Patients with nonthyroidal illness. We assayed samples from 12 patients with chronic renal failure who were receiving treatment by dialysis and from 20 patients from a general-medical ward. The latter group included patients with cardiovascular, gastrointestinal, hepatic, or respiratory disease. All of the patients with chronic renal failure and 10 patients from the medical ward had low serum T4 concentrations.

Results

Analytical Variables

Precision. The mean intra-assay precision profiles (Figure 1) were calculated from results for duplicate sample analyses from 11 SimulTRAC assays (*10*). The FT4 assay had excellent precision over a wide concentration range. The TSH assay was less precise, with a mean CV of 10% at 1.0 milli-int. unit/L.

The interassay precision data (Table 1) were calculated from duplicate analyses of pooled patients' sera and of Lyphochek control sera (Bio-Rad Labs., Richmond, CA; lot no. 07300) in 11 assays. The average bias for the Lyphochek controls was calculated from the quoted values. For the results for the in-house pools, we calculated bias from target values established by the Amerlex-M FT4 assay (Amersham International, Amersham, U.K.) for pools 1 and 2 and by an in-house TSH assay (*1*) for pools *a*–*c*. We diluted Lyphochek III and pool *c* fivefold in zero standard before measuring TSH.

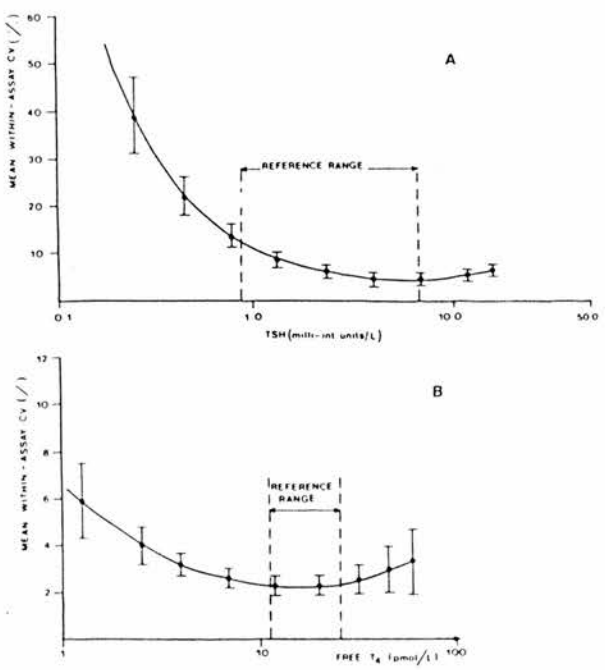

Fig. 1. Intra-assay precision profiles (mean ± SD, 11 assays) for TSH (*A*) and FT4 (*B*) by SimulTRAC RIA

Table 1. Interassay Precision of the SimulTRAC FT_4/TSH Assay

	Lyphochek			Pooled sera		
	I	II	III	1	2	
FT_4, pmol/L						
Mean	3.2	9.7	38.9	25.6	41.0	
Target	3.3	9.3	37.3	15.0	40.0	
Bias, %	−3	+4	+4	+4	+2	
SD	0.2	0.4	0.9	1.0	2.6	
CV, %	5.8	4.2	2.3	6.2	6.4	
TSH, milli-int. units/L						
	I	II	III	a	b	c
Mean	1.5	10.9	30.2	3.0	9.0	42.9
Target	1.5	11.2	23.2	2.8	7.5	44.8
Bias, %	0	−3	+30	+7	+20	−4
SD	0.1	1.0	2.7	0.2	0.5	2.2
CV, %	10.2	9.4	8.8	7.7	6.1	5.2

n = 11.

Detection limit of the TSH assay. The detection limit determined conventionally from the 95% confidence limit for 20 replicate estimations of the zero standard, was 0.1 milli-int. unit/L. Because patients' samples are not assayed in replicates of 20, a more meaningful estimate of 0.25 milli int. unit/L was obtained from the mean precision profile (Figure 1*A*) at a CV of 40%, which is equivalent to 2.5 SI from the zero standard (*10*).

Analytical recovery of TSH. Percentage recoveries of TSH IRP standard (1, 4, and 20 milli-int. units/L final concentration) added to pooled euthyroid serum were 113, 120, and 97%, respectively.

Correlation with other methods. Regression analysis of FT4 results obtained by SimulTRAC (y) and another analog FT4 RIA (Amerlex-M, x) for 32 patients' samples analyzed in the routine diagnostic laboratory because of suspected alterations in protein binding (range of FT4 results 4–3 pmol/L) gave the equation:

$$y = 1.13x - 1.7 \text{ pmol/L } (S_{yx} = 1.9)$$

Comparison of SimulTRAC TSH (y') with our in-house method (x') for values less than 10 milli-int. units/L (n = 5) gave the regression equation:

$$y' = 1.08x' - 0.44 \text{ pmol/L } (S_{yx} = 0.5).$$

These correlations differed significantly ($p < 0.001$) from $y = x$ for the x slope and the y' intercept.

Diagnostic Efficiency

The 95% confidence intervals calculated from the data of 40 euthyroid patients were 11.1–24.1 pmol/L for FT4 and 0.6–5.7 (mean 1.8) milli-int. units/L for TSH, after logarithmic transformation of the data. These intervals were not notably different from those derived by the manufacturer from results for 146 individuals: FT4, 11.4–25.3 pmol/L; TSH, 0.9–6.7 milli-int. units/L. Use of the manufacturer's reference range for TSH minimized the overlap between the different categories of patients (Figure 2).

All hyperthyroid patients (overt and subclinical) had TSH values <0.9 milli-int. unit/L and all hypothyroid patients (overt and subclinical) had TSH values >6.7 milli-int. unit/L

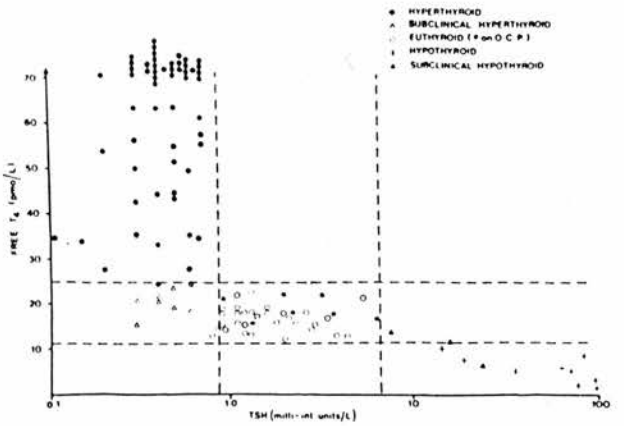

Fig. 2. SimulTRAC FT4/TSH results for 116 endocrine clinic patients, including seven women taking an estrogen-containing oral contraceptive (*O.C.P.*)
Reference limits: ---

L. With these reference limits, results overlapped for only two euthyroid patients, who had low concentrations of TSH (0.6 and 0.8 milli-int. unit/L).

All patients with overt hyperthyroidism had FT4 values exceeding 24.1 pmol/L, and all those with overt hypothyroidism had FT4 values <11.1 pmol/L. All but two patients with subclinical disease had FT4 concentrations within the reference interval: one patient with subclinical hyperthyroidism had a FT4 value of 24.8 pmol/L, and one with subclinical hypothyroidism had a low result for FT4 (6.9 pmol/L). All euthyroid patients whose T4 concentrations were increased owing to an estrogen-induced increase in TBG, had normal results by SimulTRAC. Compared with our standard strategy for investigating patients with possible thyroid dysfunction, the SimulTRAC assay produced similar categorization in 112/116 (96.5%) of the patients studied.

SimulTRAC FT4/TSH results for TSH in pregnant women were significantly (p <0.0001) higher (mean 4.0 milli-int. units/L, range 1.7–6.3) than in the nonpregnant euthyroid patients, but none exceeded the upper reference limit (Figure 3). FT4 was low in 18.5% of the women studied, and

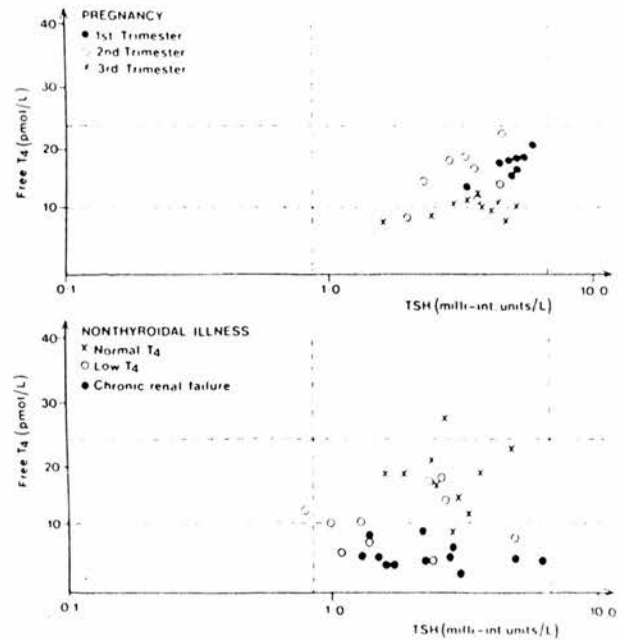

Fig. 3. SimulTRAC FT4/TSH results for pregnant women and patients with nonthyroidal illness

mean values in the third trimester, 10.8 (SD 1.5) pmol/L, were significantly lower than values for women in the first (p <0.0001) and second (p <0.01) trimesters and for nonpregnant euthyroid individuals (p <0.001).

For patients with nonthyroidal illness, TSH values were slightly higher than in euthyroid endocrine-clinic patients (mean 2.3 milli-int. units/L, range 0.8–6.3, p <0.05) but none exceeded the upper reference limit (Figure 3). In patients with normal T4 values, SimulTRAC FT4 results did not differ significantly from those for euthyroid patients attending the endocrine clinic, whereas those with low T4 values or with chronic renal failure had significantly lower FT4 values (p <0.001). Results for FT4 were below the reference interval in all 12 patients with chronic renal failure and in four of the 10 patients from the general medical ward who had low T4 concentrations.

Discussion

The SimulTRAC FT4/TSH assay is easy to perform, has acceptable reproducibility, and gives results similar to those from other analog one-step FT4 assays (*11*) and TSH RIA methods. The mean detection limit of 0.14 milli-int. unit/L, derived conventionally for the TSH assay, compares favorably with values achieved by "high sensitivity" immunoradiometric assays (*12*), but not by the new generation of nonisotopic TSH assays (*13*). In the SimulTRAC RIA, high sensitivity is achieved, to some extent, at the expense of a narrow working range. With use of appropriate curve-fitting procedures, the top standard probably could be included, extending the TSH range to 40 milli-int. units/L. The variable bias figures for TSH at high concentrations (Table 1) may be ascribable to the predilution of samples with TSH >15 milli-int. units/L rather than to poor analytical recovery.

For patients attending an outpatient endocrine clinic, the dual-analyte assay produced the same categorization as did our present investigative strategy in 96.5% of cases. This was achieved by using individual reference intervals for FT4 and TSH without recourse to the more-complex linear discriminant analysis that has been applied in the past to combinations of older thyroid-function tests (*14*). All patients with a flat result for the thyroliberin-stimulation test had basal SimulTRAC TSH values of <0.9 milli-int. unit/L. However, the fact that low TSH results were also found in 5% of euthyroid patients means that, unlike TSH measurement by more specific and sensitive assays (*15, 16*), SimulTRAC basal TSH could not be used alone as a predictor of the results of the stimulation test. Used in combination with the FT4 result, however, it allowed all patients with overt hyperthyroidism to be distinguished from euthyroid patients.

In this study, we had no patients with "T3-thyrotoxicosis"—i.e., normal values for total T4 but above-normal values for T3. Most such patients also have increased FT4 concentrations (*11*) and therefore are unlikely to be misclassified by the SimulTRAC assay. Use of this assay could therefore decrease the need for thyroliberin-stimulation tests to those patients with normal FT4 but low TSH results, in order to distinguish euthyroid patients from those with subclinical hyperthyroidism.

The 40% decline we observed in FT4 during pregnancy agrees with that seen on use of other one-step analog assays, which appear to exaggerate the decrease as measured by equilibrium dialysis (*17*) and necessitates use of trimester-related reference ranges. The presence of a normal TSH

concentration with the SimulTRAC assay confirms euthyroidism in those with low FT4. The TSH results for our pregnant women were significantly higher than in nonpregnant subjects. This may reflect a residual cross-reactivity problem with the TSH assay, despite absorption of the antiserum with human choriogonadotropin and the negligible cross reactivity with this hormone, quoted by the manufacturers, of $(1.64 \times 10^{-6})\%$.

Patients with nonthyroidal illness commonly had low FT4 results, particularly those with low total T4 concentrations. As with other analog FT4 RIAs, changes in albumin concentration and the presence of drugs and binding protein inhibitors in the serum of these patients may alter the protein-binding of the analog compared to standards, leading to low results (*18, 19*). However, none of our patients had increased TSH concentrations by SimulTRAC, so this would minimize the possibility of misdiagnosis of hypothyroidism in this group. Increases in TSH have been described during the recovery phase after systemic illness (*20*), which may account for the slightly higher TSH values in this patient group as compared with those attending the endocrine clinic.

Spuriously high results for FT4 by analog assay have been reported in rare cases, such as patients with high-affinity albumin binding (*21*) or autoantibodies to T4 (*22*). We used the SimulTRAC kit to analyze serum from two patients with such abnormalities and found normal TSH concentrations with a modest increase in FT4 (28.4 pmol/L) in a patient with high-affinity albumin, and a gross increase (FT4 >70 pmol/L) in a case with autoantibodies to T4. This combination of SimulTRAC results in an apparently euthyroid individual should alert the clinician to the possible presence of an abnormal binding protein in serum. In such patients, hyperthyroidism can be excluded by the demonstration of a normal result in a thyroliberin-stimulation test.

We conclude that the combined FT4/TSH test offers advantage over our current strategy (T4, T3, TSH, or stimulation test) by minimizing the need for thyroliberin-stimulation tests and the total number of analyses while providing the same diagnostic information. TSH measurement provides a better initial test of hypothyroidism than assay of total T4, both in outpatient clinics and in the hospital ward and, together with FT4 measurement, provides a useful means for monitoring the adequacy of thyroxin replacement therapy. For laboratories with small workloads and those cautious about adopting a supersensitive TSH measurement as their firstline thyroid-function test, measurement of FT4 and TSH by dual RIA provides a suitable alternative with advantages to the laboratory and the clinician in terms of time savings and simultaneous reporting of two complementary thyroid-function test results.

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A sensitive immunoradiometric assay for serum thyroid stimulating hormone: a replacement for the thyrotrophin releasing hormone test?

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Abstract

The value as a thyroid function test of a new, rapid, and highly sensitive immunoradiometric assay for thyroid stimulating hormone (TSH) was assessed in 188 consecutive new patients with suspected hyperthyroidism. The diagnosis was made on clinical grounds and on the basis of serum total triiodothyronine and thyroxine concentrations and the response of TSH to thyrotrophin releasing hormone (TRH) as measured by radioimmunoassay. In all except one patient the basal TSH concentration by immunoradiometric assay predicted the response of TSH by radioimmunoassay to TRH, an undetectable value being recorded in patients with a subnormal response and a measurable value in those with a normal test result. This clear relation was not observed for basal TSH concentrations as measured by radioimmunoassay. In a series of 39 hospital inpatients with acute or chronic non-thyroidal illness, of whom 11 had low concentrations of total thyroxine or triiodothyronine, or both, basal TSH concentrations were detectable by both radioimmunoassay and immunoradiometric assay in all cases and were associated with normal responses to TRH.

The immunoradiometric assay for TSH, which is commercially available, may therefore obviate the need for the more time consuming TRH test and simplify the approach to thyroid function testing in patients with suspected hyperthyroidism.

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Introduction

Measurement of the basal serum concentration of thyroid stimulating hormone (TSH) is an essential test in the investigation of suspected hypothyroidism. A raised concentration confirms a primary cause of the disease, while a normal or low concentration excludes it or, more rarely, indicates a pituitary or hypothalamic cause. Except in thyrotoxicosis induced by TSH, which is extremely rare, the basal serum TSH concentration is suppressed in hyperthyroidism. These low TSH concentrations, however, are of little diagnostic value as most

radioimmunoassays for TSH have limited sensitivity (detection limits 1-2 mU/l), so that concentrations are recorded as undetectable not only in hyperthyroid patients but also in some euthyroid patients. Measurement of serum TSH concentrations before and after intravenous administration of thyrotrophin releasing hormone (TRH) is therefore often required to discriminate between euthyroidism and hyperthyroidism. A normal TSH response excludes a diagnosis of hyperthyroidism, while a subnormal response is consistent with overt or subclinical hyperthyroidism.

The development of a sensitive immunoradiometric assay for TSH (Boots-Celltech Sucrosep TSH IRMA, Boots-Celltech Diagnostics Limited, Slough SL1 4ET, Berkshire) that is claimed to detect TSH concentrations as low as 0·05 mU/l raised the possibility that the assay might discriminate between basal concentrations in euthyroid and hyperthyroid patients, thereby avoiding the need for a TRH test. We describe our experience in assessing this possibility.

Patients and methods

We studied 188 new patients consecutively referred to a thyroid clinic with suspected hyperthyroidism. Their mean age was 48 (range 19-81). Thyroid state was assessed clinically by one of us (ADT) and by measurement of serum concentrations of total triiodothyronine, total thyroxine, and TSH (by radioimmunoassay) before and 20 minutes after intravenous administration of 200 μg TRH (TRH test). Basal TSH concentrations were also measured by the immunoradiometric assay, but the results were not used to determine the diagnostic categories of the patients. In 110 patients there was evidence of hyperthyroidism with raised serum concentrations of total triiodothyronine and thyroxine and a subnormal TSH response to TRH. The hyperthyroidism was due to Graves' disease, multinodular goitre, and solitary autonomously functioning thyroid nodule in 87, 20, and three patients respectively. Sixteen patients were considered to have subclinical hyperthyroidism on the basis of normal thyroid hormone concentrations and a subnormal TSH response to TRH. Ten of these patients had a multinodular goitre, one a solitary thyroid nodule that resolved spontaneously, two ophthalmic Graves' disease, and three diffuse thyroid enlargement presumed to represent Graves' disease in remission. Of the remaining 62 euthyroid patients, 13 had a solitary thyroid nodule, 14 a multinodular goitre, and 35 no evidence of thyroid disease. In these 62 patients normal concentrations of total triiodothyronine and thyroxine were associated with a normal TRH test result.

The above thyroid function tests were also performed within 36 hours after admission in 39 patients admitted consecutively as an emergency to a general medical unit with acute or chronic non-thyroidal illness. Their mean age was 61 (range 15-86), and none had clinical evidence or a history of thyroid disease.

After collection of blood the separated serum was stored at −20°C in aliquots for each assay. Serum total triiodothyronine and thyroxine concentrations were measured by double antibody radioimmunoassays,[1] the normal ranges being 1·1-2·8 nmol/l (0·7-1·8 ng/ml) and 70-150 nmol/l (5·4-11·7 μg/100 ml) respectively. Serum TSH concentration was measured by the double antibody radioimmunoassay routinely used in the laboratory,[2] the normal range being from undetectable to 6 mU/l. The assay used a sheep anti-TSH antiserum of good specificity such that there was no interference from follicle stimulating hormone and luteinising hormone at postmenopausal concentrations, or from human chorionic gonadotrophin at the concentrations found in pregnancy.

TSH was also measured by the Boots-Celltech Sucrosep TSH immunoradiometric assay using the protocol enclosed with the kit. Both the radioimmunoassay and the immunoradiometric assay were calibrated in terms of the TSH International Reference Preparation 68/38. The immunoradiometric assay had a substantially lower limit of detection, wider working range, and better within assay precision than the radioimmunoassay (table I). Although our radioimmunoassay has a lower limit of detection than many others, this is achieved only at the expense of a relatively long assay time. There was an excellent correlation between TSH concentrations measured by radioimmunoassay and by immunoradiometric assay in a separate series of 108 patients in whom values lay between 0·9 and 130 mU/l ($r = 0·99$; $p < 0·001$). There was no evidence of interference in the immunoradiometric assay from follicle stimulating hormone and luteinising hormone at postmenopausal concentrations, as shown by basal TSH concentrations that were consistently undetectable in both premenopausal and postmenopausal patients with thyrotoxicosis. This was in agreement with the kit manufacturer's claim of cross reactivities of less than 0·002% for both follicle stimulating hormone and luteinising hormone.

TABLE I—*Comparison of laboratory performance of radioimmunoassay and immunoradiometric assay for TSH (mean values for 10 assays)*

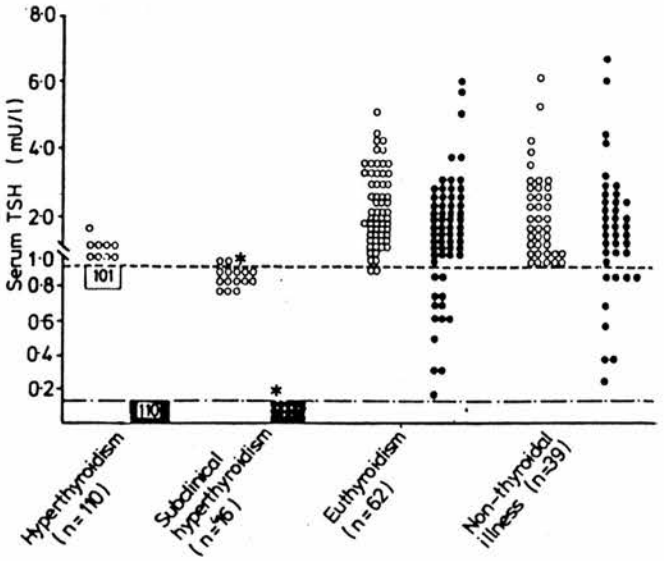
	Radioimmunoassay	Immunoradiometric assay
Detection limit (mU/l)*	0·9	0·07
Range (mU/l) for a 10% coefficient of variation	1·0-10·0	0·5-240
Within assay precision (coefficient of variation)†	5%	3%
Between assay precision (coefficient of variation)‡	7%	9%
Time required (days)	4	1

*Minimum concentration that can be distinguished from zero at 95% confidence level.
†Mean value from sample replicates.
‡Mean value for serum pools 3·0, 7·1, 21·0, and 52·0 mU/l.

Results

The figure shows basal TSH concentrations measured by both radioimmunoassay and immunoradiometric assay in the patients attending the clinic and in those with non-thyroidal illness. Among the 110 patients with hyperthyroidism TSH was undetectable by radioimmunoassay (less than 0·9 mU/l) in 101, and in the nine remaining patients concentrations of 1·0-1·7 mU/l were recorded, which overlapped the euthyroid range. In contrast, the more sensitive immunoradiometric assay recorded undetectable concentrations (less than 0·07 mU/l) in all patients. Among the 16 patients with subclinical hyperthyroidism radioimmunoassay recorded undetectable TSH concentrations in 13 and concentrations of 1·1, 1·0, and 1·0 mU/l in three; whereas immunoradiometric assay recorded undetectable concentrations in all but one patient, in whom a value of 0·14 mU/l was recorded (see Discussion). TSH concentrations were detectable in 60 of the 62 euthyroid patients and in all 39 with non-thyroidal illness when measured by radioimmunoassay, and in every patient in these two groups when measured by immunoradiometric assay.

Comparison of basal TSH concentrations with the response of TSH to TRH measured by radioimmunoassay (table II) showed that all but one patient with a detectable basal TSH concentration on immunoradiometric assay yielded a normal result to the TRH test.



Serum TSH concentrations measured by radioimmunoassay (O) and by immunoradiometric assay (●) in patients with thyroid disease and patients with non-thyroidal illness. Broken lines indicate limits of detection for radioimmunoassay (0·9 mU/l) and immunoradiometric assay (0·07 mU/l). The asterisk indicates the single patient in whom the immunoradiometric assay did not predict the result of the TRH test.

Conversely, in all patients with an undetectable basal TSH concentration on immunoradiometric assay there was a subnormal response of TSH to TRH on radioimmunoassay. The same close relation was not seen, however, between basal TSH concentrations measured by radioimmunoassay and the result of the TRH test. Thus two euthyroid patients with undetectable TSH concentrations on radioimmunoassay gave normal responses to TRH, and nine

TABLE II—*Relation between basal serum TSH and response of TSH to TRH (figures show numbers of patients in each category)*

Response to TRH	Basal TSH			
	Radioimmunoassay		Immunoradiometric assay	
	Detectable	Undetectable	Detectable	Undetectable
Normal*	60	2	62	0
Subnormal	12	114	1	125

*TSH concentration (by radioimmunoassay) at 20 minutes of 1·0 mU/l or more above basal value.

hyperthyroid patients and three patients with subclinical hyperthyroidism with detectable TSH concentrations on radioimmunoassay showed a subnormal response to TRH.

In all 39 patients with non-thyroidal illness there were detectable TSH concentrations on radioimmunoassay and immunoradiometric assay associated with normal TRH test results, despite low concentrations of total thyroxine or triiodothyronine, or both, in 11.

Discussion

The important advantages of the immunoradiometric assay compared with the radioimmunoassay—namely, higher specificity, lower limit of detection, wider working range, and speed of analysis—are a consequence of the fundamentally different principles on which the assays are based. The Boots-Celltech Sucrosep immunoradiometric assay uses a combination of two monoclonal antibodies, each of which is specific for a different epitope on the TSH molecule. One antibody is bound to a solid matrix while the other is radiolabelled. Simultaneous binding of both antibodies to TSH results in the formation of a radiolabelled insoluble "sandwich," the concentration of which is directly related to the amount of TSH in the sample. The concentration of the insoluble sandwich is indicated by the amount of radiolabel in the insoluble form. The specificity derives from the requirement for binding by two distinctly separate antibodies, while the other advantages of low limit of detection, wide working range, and speed derive from the use of excess reagents in the assay.

Although the immunoradiometric assay is superior to the radioimmunoassay from the laboratory point of view, it is important to assess whether these analytical advantages provide real improvements for the investigation of thyroid disease. Our results show that TSH concentrations as measured by the immunoradiometric assay can reliably predict the response to TRH and thereby simplify the assessment of thyroid function in patients with suspected hyperthyroidism. Whereas basal TSH concentration as measured by radioimmunoassay predicted the response of TSH to TRH in 175 out of 188 patients, when measured by immunoradiometric assay it predicted the response in 187 of the 188 patients. The exception was a patient who presented with a solitary thyroid nodule that regressed spontaneously after three months, suggesting a diagnosis of haemorrhage into a cyst or adenoma. At presentation total triiodothyronine and thyroxine concentrations were in the middle of their respective normal ranges at 1·6 nmol/l (1·0 ng/ml) and 91 nmol/l (7·1 μg/100 ml). The basal TSH concentration on radioimmunoassay was 1·0 mU/l and showed a marginally subnormal increase to 1·9 mU/l after administration of TRH. Such a result was not expected on clinical grounds, and the basal TSH concentration of 0·14 mU/l as measured by immunoradiometric assay was more consistent with the probable diagnosis.

Our findings suggest that it may no longer be necessary to perform the time consuming TRH test in patients referred with possible hyperthyroidism and that clinical assessment and measurement of total or free triiodothyronine and thyroxine concentrations and of basal TSH concentrations by immunoradiometric assay are sufficient. Like a subnormal response of TSH (on radioimmunoassay) to TRH, an undetectable TSH concentration on immunoradiometric assay would be consistent with a diagnosis of hyperthyroidism but would also be recorded, as in this series, in patients with subclinical hyperthyroidism associated with multinodular goitre, autonomous solitary nodule, and ophthalmic Graves' disease.[3] Undetectable concentrations are also likely to be recorded in some patients receiving replacement treatment with thyroxine and in the presence of normal or even low concentrations of thyroid hormones in the early weeks after treatment of hyperthyroidism due to the delayed recovery of the previously suppressed pituitary thyrotrophs.[4] A detectable result on immunoradiometric assay would exclude hyperthyroidism. It is important to consider the possible effects of non-thyroidal illness on immunoradiometric assay of basal TSH concentrations. It is well recognised that low thyroid hormone concentrations and a reduced serum TSH response to TRH may occur in patients with non-thyroidal illness, such as pneumonia, myocardial infarction, or renal failure,[5] and such abnormalities are a common cause of difficulty in interpreting test results in hospital patients. In all 39 cases in this series, however, detectable basal TSH concentrations on immunoradiometric assay correctly predicted a euthyroid state as defined by a normal response of TSH to TRH.

Immunoradiometric assays are now increasingly being used to measure peptide hormones. Although these new methods are known to have several analytical advantages over radioimmunoassay, we believe that this study is the first to show an appreciable clinical benefit from the new generation of immunoassay technology.

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A NEW STRATEGY FOR THYROID FUNCTION TESTING

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Summary In view of the increasing number of in-vitro tests of thyroid function, rationalisation of the biochemical assessment of patients with suspected thyroid disease was attempted. In addition to clinical examination of 285 consecutive new referrals to a thyroid clinic, measurements were made of serum total and free triiodothyronine (T_3) and thyroxine (T_4) and of thyrotropin (TSH) by radioimmunoassay before and 20 min after thyrotropin-releasing hormone (TRH) and basal TSH by immunoradiometric assay (IRMA). Analysis of these results demonstrated that: (i) a detectable and normal TSH (IRMA) result indicates that the patient is euthyroid and obviates the need for measurement of thyroid hormones and (ii) a raised or undetectable TSH (IRMA) level should be followed by measurement of free T_4 (and rarely also free T_3) to distinguish between subclinical and overt hypothyroidism and hyperthyroidism. This policy would considerably reduce the number of in-vitro thyroid function tests without resulting in either a delay in diagnosis or a reduction in its accuracy.

Introduction

THE diagnosis of thyroid dysfunction is based on clinical examination supported by appropriate in-vitro and, less often, in-vivo tests. Since the early 1970s, the clinician has had access to some, or all, of the following serum tests: triiodothyronine uptake test, free thyroxine (T_4) index, total T_4 (TT_4), total T_3 (TT_3), TT_4/thyroxine-binding-globulin ratio, free T_4 (fT_4), free T_3 (fT_3), basal thyrotropin (TSH) by radioimmunoassay (RIA), and TSH response to thyrotropin-releasing hormone (TRH). The most recently developed test is an immunoradiometric assay (IRMA) for TSH that is sensitive enough to discriminate on a basal serum sample the undetectable levels of overt and subclinical hyperthyroidism from those found in euthyroid patients, thereby making the TRH test redundant.[1] Commercial kits for these tests are expensive, and it is therefore important to assess which of those currently available should be recommended for use in laboratories without their own in-house assays.

Patients and Methods

We studied 285 consecutive new patients referred to the endocrine unit, Royal Infirmary, Edinburgh, either with suspected thyroid disease or for therapy with iodine-131. There were 247 females and 38 males (age range 13–80 years, mean 47·6). The diagnosis in each case was made on clinical grounds and on the basis of measurements of TT_4, TT_3, basal TSH (RIA), and the TSH response 20 min after the intravenous administration of 200 μg TRH. In addition to these tests, blood was taken for measurement of fT_3, and fT_4, and TSH (IRMA). These latter measurements did not contribute to the final diagnosis in any patient.

TT_3, TT_4, and TSH were measured by in-house radioimmunoassays,[2,3] in which the between-assay coefficients of variation (CV) were 6·6%, 4·9%, and 5·1% respectively. The normal range for TT_3 was 1·1–2·8 nmol/l and for TT_4 60–150 nmol/l. The upper limit of normal for basal TSH (RIA) was 5·7 mU/l, and the normal range of response 20 min after intravenous administration of 200 μg TRH was 3·9–25·3 mU/l.[4] An increment of less than 1·0 mU/l above the basal value was considered an absent response, consistent in this series with a diagnosis of overt or subclinical hyperthyroidism.

fT_3 and fT_4 were measured with radioimmunoassay using the Amerlex kits (Amersham International), for which the between-assay variations were 6% and 5% respectively in our own laboratory. The normal ranges calculated from the 97 euthyroid patients in this series were 4–8 pmol/l for fT_3 and 10–22 pmol/l for fT4. TSH (IRMA) was measured with the Sucrosep assay system (Boots-Celltech), in which the within-assay and between-assay coefficients of variations were 5% and 8·5%. The levels of TSH (IRMA) in the 97 euthyroid patients ranged from 0·14 to 5·9 mU/l (mean 1·9 mU/l), and an undetectable level was not recorded in any euthyroid patient.

Results

97 patients were euthyroid (normal TRH test using TSH measured with radioimmunoassay), and the results from these patients were used to establish the normal ranges for fT_3, fT_4, and TSH (IRMA). Hyperthyroidism was present in 147 patients (raised TT_4 or TT_3, absent TSH response to

TABLE I—ASSESSMENT OF TSH (IRMA) AS A SCREENING TEST FOR THYROID DYSFUNCTION

—	Correct	Incorrect
Exclusion of thyroid dysfunction	97	0
Indication for further test to distinguish between:		
a) subclinical and overt hypothyroidism	25	0
b) subclinical and overt hyperthyroidism	162	1

TRH), and subclinical hyperthyroidism in 16 (normal TT_4 and TT_3, absent TSH response to TRH). Primary hypothyroidism was present in 9 patients (low TT_4, raised TSH) and subclinical hypothyroidism in 16 (normal TT_4, raised TSH).

TSH (IRMA)

TSH (IRMA) alone correctly predicted the euthyroid state in all 97 patients and indicated overt or subclinical hyperthyroidism or hypothyroidism in all but 1 of the remaining 188 patients (table I).

fT_4 Compared with TT_4

Overt and subclinical hyperthyroidism.—fT_4 levels showed a greater rise than TT_4 in hyperthyroid patients. Results for fT_4 were in excess of 2 SD above the mean in 98% (compared with 92·5% for TT_4) and greater than 4 SD above the mean in 95% (compared with 57% for TT_4). Of the 11 patients with "T_3-thyrotoxicosis", in whom TT_4 levels were by definition normal, fT_4 levels were raised in 9. If diagnosis had rested on fT_4 and not TT_4 there would have been a reclassification from subclinical to overt hyperthyroidism in 9 of the 16 patients (55%) (fig 1).

Overt and subclinical hypothyroidism.—Like TT_4, fT_4 was low in all 9 patients with overt hypothyroidism and was also reduced in 2 of the 16 patients considered to have subclinical hypothyroidism.

fT_3 Compared with TT_3

Overt and subclinical hyperthyroidism.—fT_3 levels showed a greater rise than TT_3 in hyperthyroid patients. 97% of fT_3 values were in excess of 2 SD above the mean (compared with 89% for TT_3), and 90% were in excess of 4 SD above the mean (compared with 72% for TT_3). Whereas all but 3 of the 147 patients with hyperthyroidism had raised fT_3 levels, TT_3 was normal in 16, usually associated with drug treatment (eg, propranolol) or non-thyroidal illness.

Overt and subclinical hypothyroidism.—In the 9 patients with overt hypothyroidism the fT_3 level was normal in 2 and TT_3 was normal in 5. A marginally low level of fT_3 (fig 2) was found in 1 patient with subclinical hypothyroidism, whereas TT_3 levels were normal in all of this group.

fT_3 Compared with fT_4

From the above results it is evident that there is little to choose between fT_3 and fT_4 in suspected hyperthyroidism, but fT_4 is the better test for patients in whom hypothyroidism is the possible diagnosis.

Discussion

At present, measurements of total or free thyroid hormones are often used as a screening test for thyroid function.

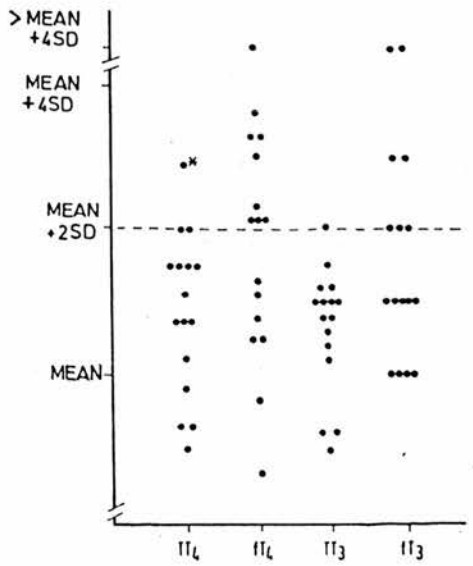

Fig 1—Thyroid hormone levels in subclinical hyperthyroidism.

*Patient taking oral contraceptive pill.

Although free hormones have advantages over total hormones in patients with binding-protein abnormalities,[5,6] they share the disadvantage of not alerting the clinician to subclinical disease. This study suggests that for practical purposes the best single screening test of thyroid function is TSH measured with a sensitive immunoradiometric assay. A normal, detectable level would indicate the euthyroid state and obviate the need for any further estimations of thyroid hormones, although in patients with goitre it might be necessary to use thyroid scanning and measurement of thyroid autoantibody titres in order to arrive at a final clinical diagnosis. If, however, a raised or undetectable level of TSH (IRMA) were recorded, it would be necessary to distinguish between overt and subclinical hypothyroidism or hyperthyroidism by measurement of thyroid hormone levels.

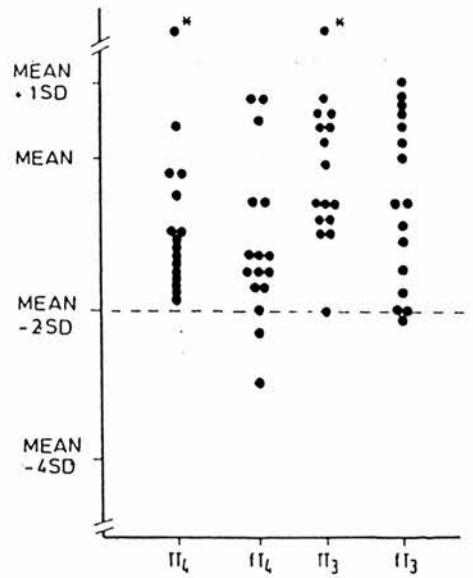

Fig 2—Thyroid hormone levels in subclinical hypothyroidism.

*Patient taking oral contraceptive pill.

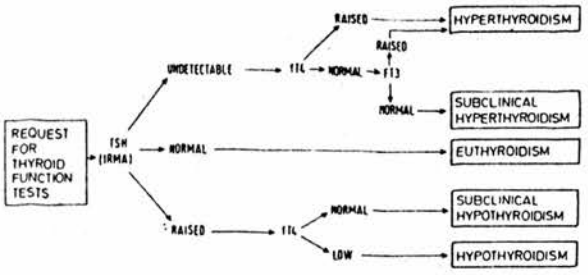

Fig 3—Proposed strategy for investigation of thyroid function.

Which hormone should be measured to define thyroid status in patients with abnormal TSH (IRMA) results? As total hormone measurements were used in this study to allocate patients to diagnostic categories, a direct comparison of the performance of free and total T_3 and T_4 is not appropriate. However, a higher proportion of free-hormone results, compared with total-hormone results, were abnormal in overt thyroid dysfunction, as reported in other studies.[7,8] It could be argued that fT_3 should be used to separate subclinical from overt hyperthyroidism and that fT_4 is the measurement of choice in the presence of a raised TSH (IRMA). However, the majority of patients with so-called T_3-thyrotoxicosis (normal TT_4 but raised TT_3 levels) were found in this study to have raised fT_4 values. It would therefore seem appropriate to use fT_4 as the arbiter of thyroid status in the presence of an abnormal TSH (IRMA) result. The strategy we propose is shown in the form of an algorithm in fig 3. The need to measure fT_3 would arise in about 3% of cases. The routine use of fT_4 in patients with an undetectable or raised TSH (IRMA) will tend to reduce the proportion of patients with subclinical disease (figs 1 and 2). This is not clinically important, because in patients with subclinical disease the natural history is progression to overt disease[9,10] and there is an attraction in treating a disorder in anticipation of possible future morbidity. Furthermore, there is some evidence that subclinical hyperthyroidism and subclinical hypothyroidism may be associated with changes in end-organ function[11-14] and are therefore mild forms of the more easily recognised overt disease.

It must be borne in mind that the results of this study may not apply to patients with non-thyroidal illness, since we know of no large studies which have assessed the performance of TSH (IRMA) in this group of patients. However, the TRH test, and presumably TSH (IRMA), is more reliable in this context than thyroid-hormone measurements.[1,15] Even if TSH (IRMA) were falsely undetectable in a small proportion, the usual findings of low or low-normal fT_3 and fT_4 would prevent the diagnosis of hyperthyroidism, and clinical examination should normally exclude the possibility of hypopituitarism. Our proposed strategy would be inappropriate for assessing thyroid status in patients on thyroxine replacement therapy in whom the combination of undetectable TSH (IRMA) and raised fT_4 would not necessarily imply overtreatment.

TABLE II—COMPARISON OF NO OF IN-VITRO ANALYSES OF THYROID FUNCTION NECESSARY USING PRESENT AND PROPOSED STRATEGIES

–	Hyperthyroidism and subclinical hyperthyroidism	Euthyroid	Hypothyroidism and subclinical hypothyroidism	Total
No of patients	163	97	25	285
Present policy:				
Test used	TT_3, TT_4, TRH	TT_3, TT_4, TRH	TT_4, TSH	
No of analyses	652	388	50	1090
Proposed strategy				
Test used	fT_4, TSH(IRMA)	TSH(IRMA)	fT_4, TSH(IRMA)	
No of analyses	334*	98†	50	482

*fT_4 analysis required in 10 patients with undetectable TSH (IRMA) and normal fT_4 (see fig 3).
†By TSH IRMA 1 subclinical hyperthyroid reclassified as euthyroid.

At present the routine measurements of fT_3, fT_4, and TSH (IRMA) are only available commercially. There would be little or no additional expense in changing to the investigative strategy we have proposed for those laboratories which depend upon commercial kits for measurement of TT_3, TT_4, and TSH (RIA). For larger centres with in-house assays for TT_3, TT_4, and TSH (RIA), the change to the use of TSH (IRMA) supplemented when necessary by fT_4 and fT_3 might appear expensive. However, the extra cost of these new thyroid function tests may be offset in part by a considerable reduction in the number of analyses which would be required. As an illustration our TSH (RIA) has a lower detection limit than many other RIA methods, but this is only achieved at the expense of a relatively long assay time, and results are not usually available to the clinician for at least a week. It is, therefore, our current practice to measure TT_3 and TT_4 in addition to the TRH test in all patients, with the exception of those with obvious clinical hypothyroidism, as the thyroid hormone levels are available within 24–48 h. This allows appropriate treatment to be instituted, if necessary, before the result of the TRH test is available. TSH (IRMA) results should be available within 24–48 h. The number of analyses done in this series would have been reduced by about 50% if diagnosis had relied on TSH (IRMA) initially, followed by measurement of fT_4 and occasionally fT_3 (table II). This reduction of in-vitro analyses of thyroid function, combined with the fact that the TRH test is now redundant,[1] should reduce the cost differential between current practice and the use of these newer tests in those laboratories with in-house assays for TT_3, TT_4, and TSH (RIA).

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CLIN. CHEM. 33/2, 303–305 (1987)

Value and Limitations of a Highly Sensitive Immunoradiometric Assay for Thyrotropin in the Study of Thyrotroph Function

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Using a highly sensitive and specific immunoradiometric assay for thyrotropin, we studied thyrotroph function in 232 new patients referred to a thyroid clinic and in 13 patients after treatment for hyperthyroidism. Significant thyrotroph responsiveness to thyroliberin (thyrotropin-releasing hormone, TRH) was found in all patients with values for basal thyrotropin >0.1 milli-int unit/L. In no overtly hyperthyroid patient was any increment in thyrotropin recorded at 20 min after thyroliberin administration. In seven patients, four subclinically hyperthyroid and three who had received treatment, increments in thyrotropin from undetectable basal values were recorded, consistent with incomplete thyrotroph suppression. By use of assays with even higher sensitivity, one may be able to distinguish these patients from overtly hyperthyroid patients.

Additional Keyphrases: *thyroid status · thyroliberin*

A recently introduced immunoradiometric assay (IRMA) for thyrotropin (TSH) is about 10-fold more sensitive than are most radioimmunoassays (RIAs).[4] Clinically it has the advantage that the initially obtained value accurately predicts the TSH (by RIA) response to thyroliberin (TRH), obviating the need for the TRH test (*1*). The high sensitivity and specificity of the IRMA should also allow more detailed assessment of thyrotroph function. We were particularly interested to investigate thyrotroph function when basal values for TSH were between the lower detection limits of the IRMA and the RIA (0.1 and 1 milli-int. unit/L, respectively), and in the months after treatment of hyperthyroidism, to determine whether detectable basal concentrations of TSH are always associated with thyrotroph responsiveness to TRH, and undetectable concentrations always with unresponsiveness. We report our findings in 232 new patient referrals and in 13 patients after treatment of hyperthyroidism.

Patients and Methods

Group One

The study population consisted of 232 new patient referrals to our thyroid clinic: 207 women and 25 men, ages 17–80 years. In each case the diagnosis was established on the basis of clinical examination, measurement of total thyroxin, triiodothyronine, and TSH by RIA before and 20 min after intravenous administration of 200 μg of TRH. In addition, serum was stored and analyzed later for TSH (by IRMA), free thyroxin, and free triiodothyronine. In 124 patients there was evidence of hyperthyroidism: increased concentrations of total thyroxin and triiodothyronine and an absent response of TSH (by RIA) to TRH. Graves' disease was diagnosed in 106 patients, a toxic multinodular goiter in 11, and a solitary toxic nodule in seven. Six patients with a multinodular goiter and six with Graves' disease were considered to have subclinical hyperthyroidism; they had normal total thyroxin and triiodothyronine and an absent TSH (by RIA) response to TRH. Five patients were hypothyroid (above-normal TSH by RIA and low total thyroxin), four with Hashimoto's thyroiditis, and one with primary atrophic hypothyroidism. A further 11 patients with Hashimoto's thyroiditis had subclinical hypothyroidism (above-normal TSH by RIA and normal values for total thyroxin). The remaining 80 patients were euthyroid (normal total thyroxin and total triiodothyronine and normal TRH test result by RIA); in 39 of these there was no evidence of thyroid disease, 18 had a multinodular goiter, 19 a solitary thyroid nodule, three a simple goiter, and one endocrine exophthalmos.

Group Two

Twelve hyperthyroid patients (11 female, one male; 11 with Graves' disease, and one with a multinodular goiter) were seen before treatment with ^{131}I and thereafter at four-week intervals for 12 weeks. In addition, one woman with Graves' disease was studied during treatment with gradually decreased doses of carbimazole. At each attendance, in addition to clinical assessment, a TRH test was performed, with results analyzed by RIA and IRMA; the basal sample was also analyzed for total thyroxin, total triiodothyronine, free thyroxin, and free triiodothyronine.

Assays

Total thyroxin, total triiodothyronine, and TSH by RIA were measured by in-house RIAs for which the respective between-assay coefficients of variation (CVs) were 4.9%, 6.6%, and 5.1% (*2, 3*). Free triiodothyronine and free thyroxin were measured by RIAs with Amerlex kits (Amersham International, Amersham, Bucks., U.K.), for which the between-assay CVs were 6% and 5%, respectively. TSH by IRMA was measured by using the "Sucrosep" system (Boots-Celltech UK), for which the between-assay CV was 8% and the mean lower limit of detection 0.1 milli-int unit/L (*4*). Normal reference intervals established in our laboratory were: total triiodothyronine 1.1–2.8 nmol/L, total thyroxin 60–150 nmol/L, free triiodothyronine 4–8 pmol/L, and free thyroxin 10–22 pmol/L. The upper limit of the normal reference interval for TSH by RIA was 5.7 milli-int units/L; an increment of <1 milli-int unit/L by RIA 20 min after TRH administration we considered to be no response.

Results

Group One

Thyrotropin by IRMA was initially undetectable in all hyperthyroid and subclinically hyperthyroid patients. We detected no increment in TSH by IRMA 20 min after TRH administration in any overtly hyperthyroid patient. Twelve

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[4] Nonstandard abbreviations: IRMA, immunoradiometric assay; TSH, thyrotropin; TRH, thyroliberin.

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Table 1. TRH Test and Thyroid Hormone Values in Seven Patients Who Showed an Increment in TSH by IRMA from Undetectable to Measurable Concentrations

Diagnosis	Months after ^{131}I	TSH, milli-int. units/L: IRMA 0 min	IRMA 20 min	RIA 0 min	RIA 20 min	Total thyroxin (nmol/L)	Total T3 (nmol/L)	Free thyroxin (pmol/L)	Free T3 (pmol/L)
MG	—	<0.15	0.26	<1.1	<1.1	142	2.0	23.0	6.0
MG	—	<0.10	0.58	<0.7	1.0	92	2.1	19.3	7.8
MG	—	<0.13	1.30	<1.0	1.2	124	2.5	15.8	6.6
MG	—	<0.10	1.30	<0.9	1.8	106	1.6	22.8	6.2
Graves'	2	<0.14	0.28	<0.8	<0.8	94	1.1	16	4.4
Graves'	2	<0.13	0.20	<0.9	<0.9	53	0.6	8	2.4
Graves'	1	<0.13	0.30	1.4	1.4	108	1.8	15	5.9

MG, multinodular goiter; T3, triiodothyronine.

patients were subclinically hyperthyroid; in four of these, all with multinodular goiters, a small increment in TSH from undetectable values was recorded by IRMA (Table 1). In these four patients, assay of TSH by RIA gave no increment in TSH in one and increments of 0.3, 0.2, and 0.9 milli-int. unit/L in the remaining three. In the four patients in whom a small increment in TSH by IRMA was detected, the concentrations of free thyroxin and free triiodothyronine were, in general, lower than in the remaining subclinically hyperthyroid patients (Figure 1). The initial value for TSH by IRMA was detectable in all of the 96 patients who were euthyroid, subclinically hypothyroid, or hypothyroid. For patients with an initial value for TSH by IRMA >0.1 and <50 milli-int. units/L, there was a strong correlation between the increment in TSH by IRMA and the initial value for TSH by IRMA (r = 0.93, p <0.001). This includes 17 patients whose basal values for TSH by IRMA were below the lower detection limit of the RIA (1 milli-int. unit/L) and above the lower limit of detection of the IRMA (0.1 milli-int unit/L). There was a substantial increment (>1 milli-int. unit/L) in TSH by IRMA in all 17 of these patients (Figure 2), of whom six had a multinodular goiter, five a solitary nodule or cyst, one a simple goiter, one endocrine exophthalmos, and four no evidence of thyroid disease.

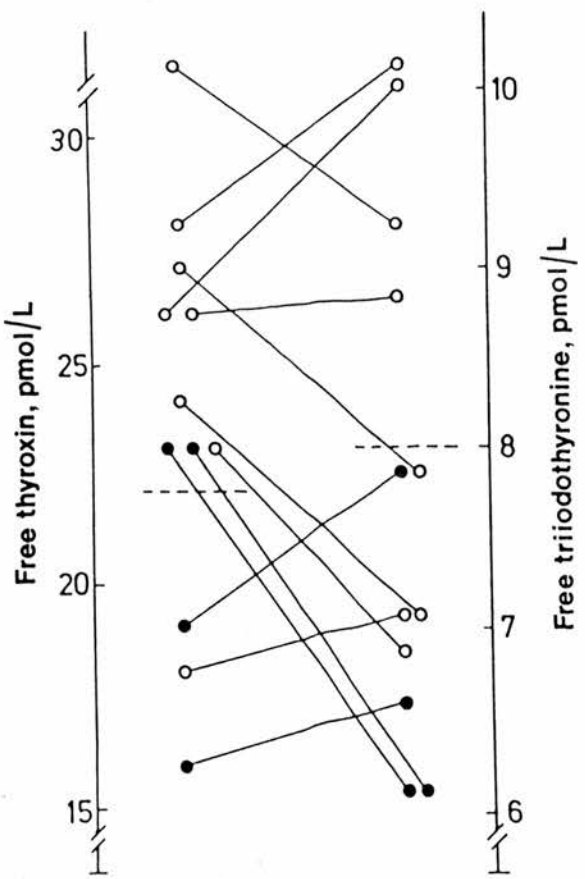

Fig. 1. Concentrations of free thyroid hormones and thyrotroph responsiveness to TRH in 12 subclinically hyperthyroid patients who did not (○) or did (●) show an increment in TSH by IRMA after TRH

Group Two

In the second group of patients, who were followed after treatment for hyperthyroidism at 12 weeks, eight were symptomatically and biochemically hypothyroid; four were clinically euthyroid with normal concentrations of thyroid hormone, but with no TSH responses to TRH by RIA and by IRMA; and one patient remained hyperthyroid.

In the eight patients who developed hypothyroidism, the response of TSH (by RIA) to TRH remained suppressed at four weeks in seven of the patients and at eight weeks in two. Three of these patients, one at one month and two at two months of treatment, showed an increase in TSH by IRMA from undetectable to detectable at a time when no increment in TSH was detectable by the RIA (Table 1). The patient treated with carbimazole had, by IRMA, after 12 weeks of treatment, a low detectable basal TSH of 0.15 milli-int. unit/L and a 20-min TSH of 1.1 milli-int. units/L, the values for TSH by RIA being 1.3 milli-int. units/L at 0 and 20 min after TRH administration.

Discussion

The highly sensitive and specific IRMA for TSH should allow for more precise study of thyrotroph function; as yet

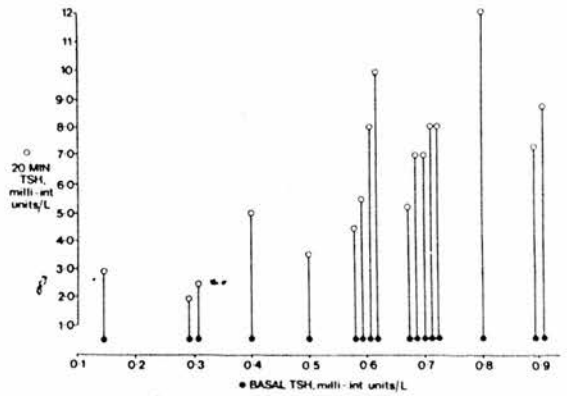

Fig. 2. TRH test results in 17 patients with basal TSH by IRMA between 0.1 and 1.0 milli-int. unit/L

●, Basal TSH; ○, TSH 20 min after TRH stimulation

however, there have been few reports of TRH tests with this new assay (*5*).

In our study, new patient referrals with an initial value for TSH by IRMA of >0.1 to <50 milli-int. units/L showed a strong correlation of the increment in TSH 20 min after TRH and the basal value of TSH by IRMA (r = 0.93). This pattern of response was observed even when the basal values for TSH by IRMA were <1 milli-int. unit/L. In these patients, therefore, the basal value for TSH by IRMA gives as much information as the TRH test concerning thyrotroph function. In hypothalamic and pituitary disease the same correlation may not hold true, but we did not have an opportunity to study such patients.

Thyrotropin by IRMA was below the detection limit of the assay in 124 hyperthyroid and 12 subclinically hyperthyroid patients at presentation; 132 showed no increment in TSH by IRMA 20 min after TRH administration. The four patients in whom an increment was detected had multinodular goiters and were considered to be subclinically hyperthyroid. Values for free thyroxin and free triiodothyronine were in general lower than in the other eight subclinically hyperthyroid patients, in whom no increment in TSH by IRMA was detected. Probably, when there is no increment in TSH by IRMA, the synthesis and secretion of TSH is completely suppressed (*6*). A small increment in TSH by IRMA from an undetectable value may indicate partial thyrotroph suppression. We suggest that, in such cases, the thyroid hormone values, although sometimes within the reference range, have risen above the usual value for that patient and have resulted in decreased synthesis and secretion of TSH. Under these circumstances a TRH test with TSH assayed by IRMA gives more information than the initial value for TSH alone. We made a similar observation in three hyperthyroid patients whose thyrotroph function returned after treatment with ^{131}I. Some patients taking thyroxin for primary hypothyroidism might also show this pattern of response.

In the study of thyrotroph function in thyroid disease, an initial value for TSH by IRMA above the detection limit of the assay gives as much information as a full TRH test; when the value is below the detection limit of the assay, however, some thyrotroph function can be demonstrated by the TRH test in a few patients. Assays for TSH of even higher sensitivity may be able to distinguish between complete and partial suppression of thyrotrophs without the need for a TRH test (*7*).

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Salivary Amylase and Pancreatic Enzymes in Sjögren's Syndrome

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Concentrations of immunoreactive trypsin (IRT) and pancreatic and salivary amylase activities were measured in 22 patients with primary Sjögren's syndrome (SS) and in 13 patients with secondary SS. Nineteen of the 22 patients with primary SS had above-normal IRT, and six had above-normal pancreatic isoamylase activity. Six of the 13 patients with secondary SS had above-normal IRT; none had above-normal isoamylase activities. Serum IRT and pancreatic isoamylase were correlated significantly (r = 0.7; p <0.0001). Above-normal values for IRT and pancreatic isoamylase were more frequent in patients who had SS for longer than 10 years, but were not related to the presence of salivary gland autoantibodies or to salivary isoamylase activity. We conclude that the concentration and activity of pancreatic enzymes are frequently abnormal in SS; that the abnormality is greater and more frequent in patients with primary SS; and that it increases with the duration of the disease.

Additional Keyphrases: *isoenzymes · trypsin · rheumatoid arthritis · systemic lupus erythematosus*

Sjögren's syndrome (SS) is characterized by progressive destruction of the salivary and lacrimal glands by a chronic inflammatory process (*1, 2*).[3] This leads to a decrease in salivary flow, dryness of the mouth and eyes, and sometimes other exocrine disturbances. SS is classically subdivided into two groups: primary SS (or sicca syndrome), where exocrine disturbances occur in isolation, and secondary SS, where a well-characterized disorder of connective tissue is also present. This clinical subdivision is supported by clinical, immunological, and immunogenetic differences between the two groups.

Two previous studies suggested that concomitant abnor-

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[3] Nonstandard abbreviations: SS, Sjögren's syndrome; RA, rheumatoid arthritis; SLE, systemic lupus erythematosus; IRT, immunoreactive trypsin.

CLINICAL RESEARCH

Raised plasma glutathione S-transferase values in hyperthyroidism and in hypothyroid patients receiving thyroxine replacement: evidence for hepatic damage

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Abstract

Using plasma glutathione S-transferase measurements hepatocellular integrity was assessed in groups of hyperthyroid and hypothyroid patients before and after treatment.

Ten of 14 hyperthyroid patients had clearly raised plasma glutathione S-transferase values at presentation and in each patient treatment with either iodine-131 or carbimazole resulted in a significant fall in glutathione S-transferase. The eight hypothyroid patients had normal glutathione S-transferase values at presentation and all showed a significant increase in these after thyroxine replacement therapy. In three of these patients in whom standard doses of replacement therapy were associated with a raised free thyroxine (T4) concentration but normal total and free triiodothyronine (T3) values glutathione S-transferase was increased. Similar though less consistent changes were seen in the results of standard chemical tests of liver function.

It is concluded that hyperthyroidism may produce subclinical liver damage in a high proportion of patients and that this resolves with effective treatment. More important, the data suggest that hypothyroid patients receiving thyroxine replacement therapy may have similar subclinical liver damage.

Patients receiving thyroxine should be monitored by the measurement of free, not total hormone concentrations, and in those in whom free T4 is raised the dose of thyroxine should be reduced. It would also be expedient to include periodic biochemical assessment of liver function in patients receiving thyroxine.

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Introduction

Before the advent of effective treatment for hyperthyroidism serious hepatobiliary complications were commonly associated with the disease. Liver biopsy samples taken from hyperthyroid patients often showed morphological changes which included glycogen depletion, fatty change, and cirrhosis. These associated hepatic problems have been attributed to many factors, including cardiac failure, infection, hypoxia, and malnutrition, and are not thought to be due to a direct effect of thyroid hormones on the liver.[1]

Prolonged and severe hyperthyroidism now occurs only occasionally and, as a consequence, severe hepatobiliary dysfunction associated with hyperthyroidism is rare. There is, however, evidence that hyperthyroidism may still produce associated minor hepatic abnormalities, and electron microscopy of liver biopsy samples shows non-specific changes in the hepatocyte organelles.[2] Biochemical evidence of hepatic dysfunction may still be seen in some patients with hyperthyroidism. In one study retention of sulphobromophthalein was abnormal in 8% of patients, and abnormalities in bilirubin and alanine aminotransferase values also were observed, but less frequently.[3]

There is little evidence to suggest that hypothyroidism affects liver function, but these patients will subsequently receive levothyroxine replacement therapy. As a result many will have raised plasma total and free thyroxine (T4) concentrations with normal total and free triiodothyronine (T3) concentrations.[4] Of particular interest is the question whether these patients, who appear euthyroid clinically, have tissue hyperthyroidism.[5] Measurement of the changes which occur in cardiac and pituitary response in these patients suggests that tissue hyperthyroidism does result from this treatment.[5] Nevertheless, thyroxine replacement therapy has not been shown to have a detrimental effect on hepatic function.

The measurement of plasma glutathione S-transferase by radioimmunoassay offers an extremely sensitive method of investigating hepatocellular damage, both in animals and man.[6-9] These measurements are non-invasive and the results correlate well with the histological findings in the liver.[7] In drug induced liver damage plasma glutathione S-transferase measurements appear to be some 10 times more sensitive at detecting damage than measurements of aminotransferase activities.[8]

We report the use of plasma glutathione S-transferase measurements and standard chemical tests of liver function to investigate the hepatocellular integrity of patients with thyroid diseases. We have also examined the effect of thyroxine replacement on hepatocellular integrity in hypothyroid patients.

Patients and methods

HYPERTHYROID PATIENTS

We studied two groups of patients with hyperthyroidism. The first group consisted of five women aged 46-75 years (cases 1-5; see table I) who were treated with iodine-131. All had raised plasma concentrations of total T4 and total T3 at presentation, and all showed a basally suppressed thyroid stimulating hormone concentration which did not rise by more than 1 mU/l 20 minutes after an intravenous injection of 200 μg thyrotrophin releasing hormone.

The second group consisted of nine younger women (aged 19-45 years; cases 6-14) in whom a study had been undertaken to assess the merits of different treatment schedules with carbimazole. These schedules were (*a*) 15 mg carbimazole three times a day for one month, subsequently reduced in the light of clinical and biochemical response, and (*b*) administration of a continuous blocking dose of 15 mg carbimazole three times a day with triiodothyronine replacement (20 μg thrice daily) being added after roughly one month to prevent hypothyroidism. As in the first group, all patients had raised total T4 and total T3 concentrations at presentation with a lack of response of thyroid stimulating hormone to injection of thyrotrophin releasing hormone.

In all patients blood was taken at presentation and plasma stored at −20°C for the subsequent measurement of total and free T4 values, total and free T3, glutathione S-transferase, aspartate aminotransferase, γ-glutamyltransferase, and alkaline phosphatase. In the first group of patients a second sample was taken when they had been rendered euthyroid. In the patients treated with carbimazole more frequent sampling was employed.

HYPOTHYROID PATIENTS

The hypothyroid group of patients comprised eight women aged 20-64 years (cases 15-22) presenting with primary atrophic (four patients) or Hashimoto's hypothyroidism. Each patient was assessed at diagnosis and at intervals from three to nine months after beginning thyroxine replacement therapy (see table II). One patient (case 15) was taking the combined oral contraceptive pill.

MEASUREMENTS

Concentrations of total T4 and total T3 were measured by radioimmunoassay using a double antibody system.[10] Free T4 and free T3 concentrations were measured with commercial kits (Amersham International PLC). Reference ranges derived from 98 clinically and biochemically euthyroid patients were: total T4, 65-145 nmol/l (5·1-11·3 μg/100 ml); total T3, 1·2-2·8 nmol/l (0·8-1·8 ng/ml); free T4, 10-22 pmol/l (7·8-17·1 pg/ml); free T3, 4·0-7·8 pmol/l (2·6-5·1 pg/ml).

Plasma glutathione S-transferase was measured by radioimmunoassay.[11] The concentration of hepatic glutathione S-transferase B_1B_1[12] (previously referred to as basic glutathione S-transferase[9]) was measured with a specific antiserum which showed no cross reactivity with the anionic forms of glutathione S-transferase found in red cells, muscle, or lung. The upper limit of the reference range for glutathione S-transferase B_1B_1 was 4·0 μg/l and was derived from 135 blood donors and 40 laboratory volunteers.

The activities of alkaline phosphatase and γ-glutamyltransferase in plasma were measured by a sequential multiple analysis with computer system (SMAC II, Technicon Instrument Corporation Basingstoke). The activity of aspartate aminotransferase was measured with a centrifugal fast analyser and a kit method (Merckotest A E Merck, Darmstadt).

The between and within batch coefficient of variation was <9% for each assay.

Statistical analysis was by Wilcoxon's matched pairs test.

Results

HYPERTHYROID PATIENTS GIVEN ^{131}I

Three of the five patients treated with ^{131}I initially had raised plasma glutathione S-transferase values. After treatment all showed a significant ($p < 0·05$) fall in values to within the reference range

TABLE I—*Liver function values in hyperthyroid patients before and after ^{131}I or carbimazole treatment and in hypothyroid patients before and after thyroxine replacement therapy. (Reference ranges given in parentheses.) For clarity abnormal values given in italics*

Case No	Before treatment				After treatment			
	Aspartate aminotransferase (10-35 U/l)	Alkaline phosphatase (40-100 U/l)	γ-glutamyltransferase (5-35 U/l)	Glutathione S-transferase (<4·0 μg/l)	Aspartate aminotransferase (10-35 U/l)	Alkaline phosphatase (40-100 U/l)	γ-glutamyltransferase (5-35 U/l)	Glutathione S-transferase (<4·0 μg/l)
				^{131}I treated hyperthyroid patients				
1	*47*	*213*	*63*	*10·2*	33	99	16	4·0
2	*39*	*270*	*101*	*6·8*	*44*	*248*	30	2·4
3	*40*	*306*	*217*	*4·4*	*36*	*377*	*86*	1·3
4	30	*172*	29	3·5	*38*	*133*	15	1·6
5	24	91	10	2·4	18	*132*	9	1·8
				Carbimazole treated hyperthyroid patients				
6	*44*	42	*37*	*6·2*	23	51	12	3·7
7	23	*117*	23	*9·8*	18	*127*	9	3·8
8	27	*111*	18	*8·4*	19	82	6	3·9
9	29	73	10	*10·0*	23	88	8	4·0
10	25	60	10	*10·0*	18	71	10	*7·0*
11	32	*110*	*36*	*11·0*	21	*118*	12	*8·8*
12	21	89	9	*9·4*	21	50	15	*6·3*
13	24	73	16	*5·2*	24	87	10	3·8
14	24	61	13	*9·3*	28	89	9	*14·0*
				Thyroxine treated hypothyroid patients				
15	16	40	5	1·0	28	46	6	1·0
16	20	65	15	1·2	23	80	17	2·3
17*	23	56	17	1·7	23	59	*41*	7·9
18*	35	95	*41*	2·6	*43*	94	*94*	*15·6*
19	17	44	9	1·5	21	70	8	3·0
20	33	99	<5	2·4	*72*	*125*	25	*11·4*
21	28	*106*	16	1·7	35	*109*	14	2·9
22*	26	53	<5	2·3	21	77	8	7·7

*Patient with raised free T4 concentration after replacement therapy.

(fig 1). In one patient (case 1) the glutathione S-transferase value was at the upper limit of the reference range after treatment, but in this patient the ^{131}I had produced hypothyroidism necessitating thyroxine replacement therapy.

Table I gives the results of the standard liver function tests. The three patients in whom glutathione S-transferase was raised also showed abnormalities in aspartate aminotransferase, alkaline phosphatase, and γ-glutamyltransferase activities. In addition, one further patient had an isolated increase in alkaline phosphatase activity. Patients did not show a significant fall in aspartate aminotransferase or alkaline phosphatase after treatment, but γ-glutamyltransferase activities did fall significantly ($p < 0.05$) with treatment.

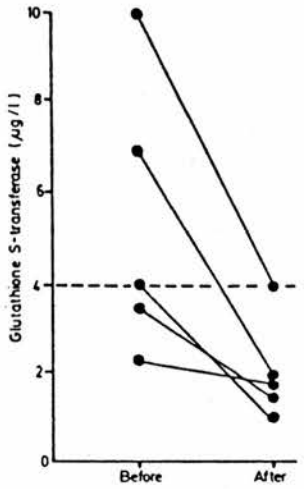

FIG 1—Plasma glutathione S-transferase measurements in five patients before and after treatment with ^{131}I for hyperthyroidism. Dashed line represents upper limit of reference range.

HYPERTHYROID PATIENTS GIVEN CARBIMAZOLE

All patients treated with carbimazole initially had raised plasma glutathione S-transferase values. After treatment all showed a significant ($p < 0.01$) fall in values. Although the fall in plasma glutathione S-transferase tended to parallel the fall in total and free T4 concentrations, equivocal or raised glutathione S-transferase values were still found up to five months after the start of treatment (fig 2). No significant difference in glutathione S-transferase values after treatment were observed between the different carbimazole treatment regimens.

In several patients there was a transient rise in glutathione S-transferase after the initiation of treatment (fig 2). In one (case 14), who showed a sensitivity reaction of a skin rash and arthralgia, the rise was pronounced. In this patient subsequent treatment with propylthiouracil produced a similar rise in glutathione S-transferase with a concomitant sensitivity reaction (fig 3). In this patient treatment was subsequently changed to ^{131}I.

Although glutathione S-transferase values were raised in all patients before treatment, only four showed abnormalities in standard liver function values (table I). After treatment with carbimazole there was a significant ($p < 0.05$) fall in activities of aspartate aminotransferase and γ-glutamyltransferase, normal values being found in all treated patients.

PATIENTS WITH HYPOTHYROIDISM

Each of the eight patients with hypothyroidism initially had normal plasma glutathione S-transferase values (fig 4) and all had raised concentrations of thyroid stimulating hormone and low concentrations of total and free T4. The concentration of total or free T3 was

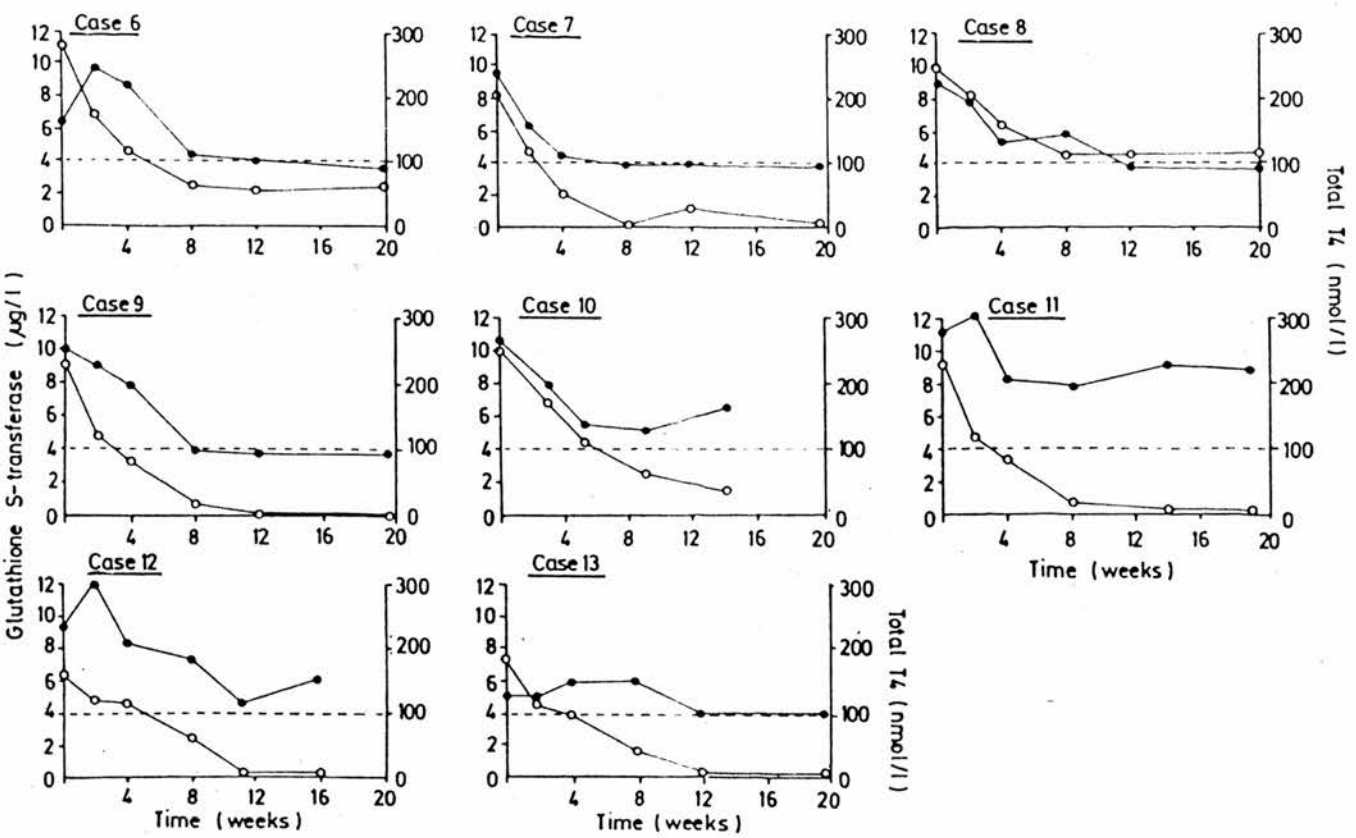

FIG 2—Sequential plasma measurements of total T4 (○) and glutathione S-transferase (●) in eight patients treated for hyperthyroidism with carbimazole. Three patients (cases 6-8) had dose of carbimazole reduced from 15 mg thrice daily in light of clinical and biochemical response. Remaining patients received continual blocking dose of carbimazole (15 mg thrice daily) and began triiodothyronine replacement (20 μg) after one month. Dashed lines represent upper limit of reference range for glutathione S-transferase. Reference range for total T4, 65-145 nmol/l.

Conversion: SI to traditional units—Total T4: 1 nmol/l ≈ 0·08 μg/100 ml.

TABLE II—*Thyroid hormone concentrations before and after thyroxine replacement therapy in eight consecutive patients with primary hypothyroidism. (Reference ranges given in parentheses)*

Case No	Before thyroxine				After thyroxine				Thyroxine dose (μg/24 h)	Duration (months)
	T4		T3		T4		T3			
	Total (65-145 nmol/l)	Free (10-22 pmol/l)	Total (1·2-2·8 nmol/l)	Free (4·0-7·8 pmol/l)	Total (65-145 nmol/l)	Free (10-22 pmol/l)	Total (1·2-2·8 nmol/l)	Free (4·0-7·8 pmol/l)		
15	43	5	1·9	5·2	112	21	1·4	5·9	100	8
16	55	7	1·6	4·2	108	17	1·3	4·1	100	8
17*	< 25	2	0·7	< 1·6	176	35	1·9	7·1	200	9
18*	27	4	1·3	3·5	136	28	1·9	7·1	150	3
19	65	9	1·5	4·6	121	19	1·3	4·2	150	8
20*	< 20	4	1·1	3·7	91	16	1·8	5·3	100	5
21	31	4	1·6	3·2	109	17	1·7	5·4	150	8
22*	< 20	< 1	< 0·5	1·9	116	31	1·8	7·7	200	6

*Patients with abnormal glutathione S-transferase value after replacement therapy.
Conversion: SI to traditional units—Total T4: 1 nmol/l ≈ 0·08 μg/100 ml. Free T4: 1 pmol/l ≈ 0·8 pg/ml. Total T3: 1 nmol/l ≈ 0·7 ng/ml. Free T3: 1 pmol/l ≈ 0·7 pg/ml.

subnormal in five of these patients (table II). While three patients showed clearly abnormal free T4 concentrations after treatment, only one showed an abnormal total T4 value (table II).

After thyroxine replacement therapy all patients showed a significant ($p < 0·01$) rise in plasma glutathione S-transferase (fig 4). In four patients clearly raised values were found, similar to those observed in the hyperthyroid patients. During thyroxine replacement three patients had raised free T4 concentrations, and in each of these

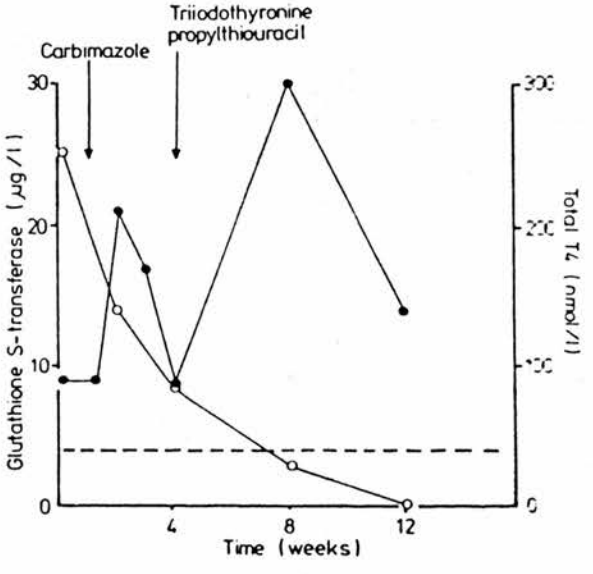

FIG 3—Case 14. Sequential total T4 (○) and glutathione S-transferase (●) measurements in patient treated for hyperthyroidism with carbimazole and subsequently with propylthiouracil and supported with triiodothyronine. After treatment with carbimazole and propylthiouracil (arrowed) pronounced sensitivity reaction occurred. Dashed line represents upper limit of reference range for glutathione S-transferase.

raised glutathione S-transferase values were found. The patient in whom free T4 was normal and glutathione S-transferase raised had been receiving long term treatment with mefenamic acid. This patient also showed abnormalities in aspartate aminotransferase and alkaline phosphatase activities after treatment with thyroxine. In all other patients normal free T4, total T4, and glutathione S-transferase values were found after treatment. All eight patients had normal concentrations of free and total T3 and either normal or suppressed concentrations of thyroid stimulating hormone. There appeared to be no association between glutathione S-transferase values after treatment and the dose or duration of thyroxine replacement therapy.

After thyroxine replacement therapy there was a significant ($p < 0·05$) increase in alkaline phosphatase activity (table I). Two of the patients with raised free T4 concentrations after treatment had abnormalities in γ-glutamyltransferase, and in one of these patients aspartate aminotransferase activity was also increased.

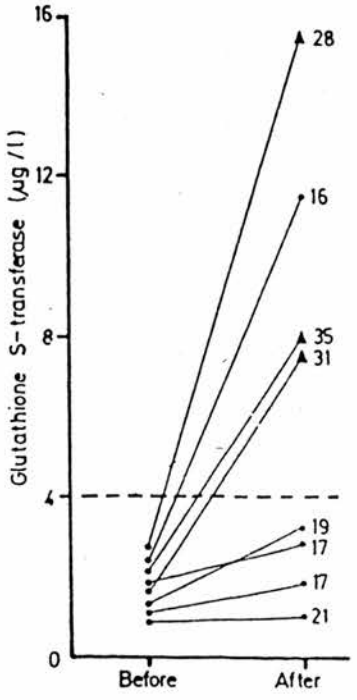

FIG 4—Plasma glutathione S-transferase measurements in hypothyroid patients before and after thyroxine replacement therapy. ▲ = Patients with raised free T4 concentrations after treatment (actual values shown in pmol/l). All patients had normal total and free T3 values after treatment.
Conversion: SI to traditional units—Free T4: 1 pmol/l ≈ 0·8 pg/ml

Discussion

It is generally thought that hepatic dysfunction is no longe a common complication of hyperthyroidism, since abnormaliti in results of the standard chemical tests of liver function a found infrequently. We,[*] however, have shown that a hig proportion of patients with untreated hyperthyroidism ha greatly increased plasma glutathione S-transferase values ar that successful treatment with ^{131}I or carbimazole results in fall in these values which roughly parallels the fall in total T concentration.

Unlike aspartate aminotransferase, plasma glutathione transferase values appear to correlate well with the histologi picture in patients with chronic active liver disease,[7] and al unlike alanine aminotransferase the incidence of abnorm glutathione S-transferase values in patients with paracetam poisoning[8] agrees well with the incidence of abnormal hist logical findings in the livers of these patients.[13] In this conte

our finding of a high incidence of raised plasma glutathione S-transferase values in hyperthyroidism agrees with the previously reported observation, with electron microscopy, that minor structural abnormalities commonly occur in the organelles of the hepatocytes of patients with this disease.[2] Our observations are therefore consistent with the hypothesis that minor hepatocellular dysfunction may still be a common though subclinical complication in hyperthyroidism.

Glutathione S-transferases are a "multigene" group of enzymes that are concerned in the cellular detoxification of a wide range of electrophiles. In human liver and kidney the main forms of the enzymes are cationic (pI 8·4-8·9), while in other tissues an anionic form (pI 4·8) predominates. These enzymes are dimeric, and in human liver two cationic subunits, B_1 and B_2, have been identified. The radioimmunoassays which we use are specific for either the B_1 or B_2 monomers and do not react with the anionic forms of glutathione S-transferase. As well as the plasma values of hepatic glutathione S-transferase B_1B_1 we also measured hepatic glutathione S-transferase B_2B_2 (previously called N/A2b).[11 12] For clarity these data have not been included but in all cases abnormal basic glutathione S-transferase B_1B_1 values were associated with abnormal values of glutathione S-transferase B_2B_2. The subunit forms of glutathione S-transferase which we have measured specifically by radioimmunoassay are confined predominantly to the hepatocyte and the renal tubules, little of these subunit forms being found in tissues such as muscle, red cells, or lung.[14]

We have considered the possibilities that the raised plasma glutathione S-transferase values found in the hyperthyroid patients were a consequence of hepatic enzyme induction by thyroxine or may merely have reflected extrahepatic tissue damage. Both these possibilities seem unlikely. Firstly, when thyroxine is administered to hypothyroid or euthyroid animals either a small decrease or no change in hepatic glutathione S-transferase is observed.[15] Secondly, as the cationic forms of glutathione S-transferase are relatively specific to the liver and kidney, and since renal damage is not found in hyperthyroidism, it appears that the abnormal plasma values of both B_1 and B_2 originate from hepatic damage.

The cause of the hepatic lesions observed in hyperthyroidism is not clear but is believed to be multifactorial, with selective and general hypoxia as contributory factors.[1] Thyroxine itself has not been reported as directly hepatotoxic. Nevertheless, our observation that prolonged administration of thyroxine to hypothyroid patients resulted in significant increases in plasma glutathione S-transferase in all patients has implications for the long term management of these patients. It is also relevant to the controversy about the importance of raised plasma free T4 concentrations in patients receiving thyroxine.

In our study all of the treated hypothyroid patients with raised free T4 concentrations had associated abnormalities in plasma glutathione S-transferase values. Our data may indicate that administration of thyroxine in doses which result in raised plasma concentrations of free T4 may produce a hepatic lesion similar to that seen in hyperthyroidism. Jennings *et al* have shown that hypothyroid patients who are receiving thyroxine replacement and in whom free and total T4 concentrations are raised have a decreased systolic time interval, which suggests that they have a "tissue thyrotoxicosis."[5] Our data suggest that they may also have a "liver thyrotoxicosis."

The thyroid gland secretes approximately 90 μg thyroxine a day into the peripheral circulation. Patients who are receiving oral replacement therapy will receive roughly this amount as a single dose. Administered in this way, high concentrations of thyroxine are presented to the liver, through the portal vein. Thus, although peripheral blood concentrations of thyroid hormone may appear satisfactory, it is possible that in these patients liver thyrotoxicosis results from an exposure to high portal concentrations of thyroxine.

Plasma glutathione S-transferase values returned to normal in hyperthyroid patients treated with ^{131}I, but in patients treated with carbimazole they remained raised even five months after treatment began. In roughly half of the carbimazole treated patients glutathione S-transferase values were increased in the first sample after treatment; in one patient this rise was pronounced and associated with physical signs of a sensitivity reaction. It appears that carbimazole may cause transient subclinical liver damage in some patients, and hepatobiliary problems associated with carbimazole, methimazole, and propylthiouracil have been reported in a few.[16-18] Our data, however, do not show that carbimazole is necessarily hazardous, since the rises in plasma glutathione S-transferase after carbimazole were transient and patients are unlikely to receive carbimazole for long periods. On the other hand, our finding that hepatic abnormalities may occur in patients receiving thyroxine replacement is of concern, since for these patients treatment will be life long. Hepatic function should therefore be considered and assessed periodically in such patients. Measurement of the plasma glutathione S-transferase value by radioimmunoassay appears to provide the most sensitive and specific method of assessing hepatic function in these patients. Nevertheless, the availability of glutathione S-transferase assays is restricted to a few centres, and it is therefore reassuring to note that three of the four hypothyroid patients who had raised glutathione S-transferase values after treatment would have been detected by the standard liver function tests. These tests may offer a satisfactory alternative to glutathione S-transferase measurements in clinical practice.

In conclusion, our data, and those of others,[5] indicate that the thyroxine dose should be lowered in patients who have raised free thyroid hormone concentrations in order that free T3 and free T4 may revert to normal.

We are grateful to Dr D B L McClelland and Dr P L Yap, of the Edinburgh and South East Scotland Regional Blood Transfusion Service, for supplying blood donor samples to derive the reference range for glutathione S-transferase. This work was supported by a Scottish Home and Health Department grant awarded to GJB and JDH. We also thank Professor L G Whitby and Dr A F Smith for help and advice in the preparation of this manuscript, and Mrs E Ward for secretarial work.

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Relationship between Pituitary and Other Target Organ Responsiveness in Hypothyroid Patients Receiving Thyroxine Replacement*

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ABSTRACT. This study was undertaken to compare the sensitivity of the thyrotrophs to that of other tissues to T_4 treatment in hypothyroid patients. To do so, we measured serum total and free thyroid hormones and TSH, in addition to several serum markers of peripheral tissue response to thyroid status, in 21 hypothyroid patients treated with 50-μg increments of T_4 to a maximum of 200 μg daily (group I) and in 104 clinically euthyroid patients receiving a long term constant replacement dose (group II).

In group I patients, dose-dependent increases ($P < 0.05$) in serum glutathione *S*-transferase, sex hormone-binding globulin, and angiotensin-converting enzyme occurred, whereas serum T_4-binding globulin, creatine kinase, and creatinine levels decreased ($P < 0.05$). In both patient groups, abnormally high levels of glutathione *S*-transferase, sex hormone-binding globulin, angiotensin-converting enzyme, alanine aminotransferase, and γ-glutamyl transferase were found in some patients during treatment. One or more of these biochemical abnormalities suggestive of hyperthyroidism occurred in 15 (71%) group I patients and 27 (26%) group II patients. These were associated with an undetectable serum TSH (<0.1 μU/ml) and raised free T_4 concentrations in 13, and raised free T_3, T_4, and T_3 concentrations in only 8, 6, and 1 group I patients, respectively. In group II patients, they were more closely associated with an undetectable TSH (67%) or raised free T_4 (85%) level than with raised concentrations of free T_3 (33%), T_4 (26%), or T_3 (0%).

The use of high sensitivity TSH assays will permit more accurate adjustment of T_4 replacement and minimize abnormalities in peripheral tissue biochemistry indicative of overtreatment. (*J Clin Endocrinol Metab* **64**: 364, 1987)

THE REPLACEMENT dose of T_4 for primary hypothyroidism has gradually been reduced from 200–400 to 100–200 μg daily as better tests for monitoring therapy have been introduced. However, overtreatment is difficult to assess by measurement of serum T_4 alone, since to achieve normal serum TSH levels, sufficient T_4 to raise serum T_4 (and free T_4) concentrations above reference limits may be needed (1). TSH measurements performed by RIA allow titration of the dose of T_4 such that TSH is no longer elevated (2, 3), but due to the poor sensitivity and specificity of most of these assays, such measurements cannot be used to identify overtreatment. It has been argued that since T_3 is the most active thyroid hormone (4), the aim of T_4 replacement therapy should be to normalize serum total or free T_3 concentrations (5, 6). When this is done, many patients have increased serum free T_4 concentrations, but since few have clinical signs of hyperthyroidism, it has been assumed that they suffer no deleterious effects. However, some such pa tients have altered responses at the tissue level, e. diminished activity of the red cell sodium pump (7, 8 histological bone changes indicative of osteoporosis (9 abnormalities in biochemical tests of liver function (10 and increases in myocardial contractility, with systol time intervals in the thyrotoxic range (11).

Recently, highly sensitive assays for serum TSH ha been developed. These assays can reliably distinguish tl undetectable (<0.1 μU/ml) levels found in thyrotox patients from normal levels (12). Many patients receivi T_4 replacement have serum TSH levels undetectable l a sensitive assay (13) consistent with their absent TS responses to TRH (1). However, the pituitary diffe from some other tissues in that T_3 derived from loc conversion from T_4 within the cell occupies a great proportion of T_3 nuclear receptors than T_3 derived fro serum (14). This greater sensitivity of the pituitary serum T_4 has led to the assumption that it is an uni presentative target tissue regarding the assessment overtreatment with T_4.

As alternatives to the more sophisticated measu ments of peripheral tissue responses described (7–9, 1

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ve have used simple measurements of constituents in serum known to be altered in patients with overt thyroid disease and related these to TSH secretion. High concentrations of alanine aminotransferase (ALT), liver-specific glutathione *S*-transferase (GST), sex hormone-binding globulin (SHBG),' and angiotensin-converting enzyme (ACE) have all been described in hyperthyroidism (10, 15–17), whereas concentrations of T_4-binding globulin (TBG) and creatinine may be decreased (18, 19). Raised concentrations of creatine kinase (CK) are found in hypothyroidism (20). Changes in the concentrations of these analytes reflect altered entry to (due to changes in synthesis or membrane permeability) or clearance from the blood. These markers are arguably crude and insensitive indicators of thyroid status which cannot serve to assess thyroid status in an individual. However, when many of these markers are measured in groups of patients with overt hyperthyroidism, unequivocal statistically significant abnormalities are found. In this study we measured these markers in groups of patients receiving T_4 replacement therapy to provide evidence of the suitability of the T_4 dose and compared the results with measurements of serum free and total thyroid hormone and TSH concentrations.

Subjects and Methods

Patients

Two groups of patients were studied. Group I consisted of 21 hypothyroid patients (17 women and 4 men; mean age, 50 yr; range, 31–68) starting T_4 replacement. The causes of hypothyroidism in this group were primary atrophic disease (n = 7), Hashimoto's thyroiditis (n = 2), and ^{131}I therapy for either Graves' disease (n = 11) or multinodular goiter (n = 1). The average length of time between radioiodine treatment and the start of T_4 treatment was 5 months (range, 2–11). The T_4 dose was increased each month by 50-μg increments to a maximum of 200 μg/day. Blood samples were taken at the end of each month. In addition, 5 group I patients had samples taken pre- and postradioiodine treatment before T_4 therapy was started.

To address the question of whether equilibration of serum analytes had occurred in 1 month, nine patients were retested after 2 additional months of treatment with the highest dose of T_4 that they had tolerated during the study.

Group II comprised 104 patients (91 women and 13 men; mean age, 51 yr; range, 17–85). Hypothyroidism was the result of ^{131}I or surgical treatment of thyrotoxicosis in 51 and 9 patients, respectively, and in the remainder was due to Hashimoto's thyroiditis (23 patients) or primary atrophic disease (21 patients). At the time of investigation all were clinically euthyroid and had been receiving a constant replacement dose of T_4 for at least 3 months (mean, 3.5 yr); the majority (>89%) had taken the same dose for more than 6 months. The largest group (n = 47) was taking 100 μg/day; the others were taking 50 μg (n = 5), 75 μg (n = 1), 150 μg (n = 28), 200 μg (n = 19), or 300 μg (n = 4) per day.

Methods

Serum total T_4 and T_3 levels were measured by in-house RIAs (21). Serum free T_4 and free T_3 levels were measured using Amerlex-M kits (Amersham International, Amersham Bucks, United Kingdom). Serum TSH was measured by an immunoradiometric assay (IRMA; Boots-Celltech, Slough, Berks United Kingdom), which has a minimum detection limit of 0.1 μU/ml. The hepatic (B_1B_1) form of GST was measured by RIA (22). Serum ALT, GGT, and creatinine were measured using a Technicon sequential multichannel analyzer with computer (Technicon Instruments Co., Tarrytown, NY). Commer-

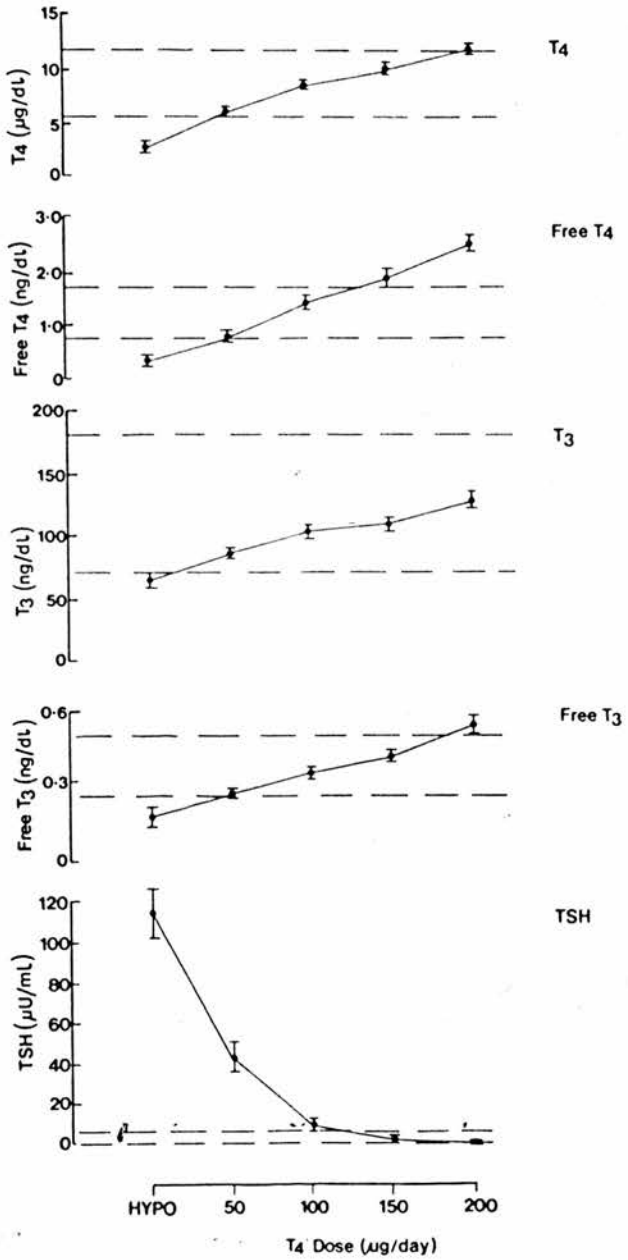

FIG. 1. Mean (±SE) thyroid function test results in 21 hypothyroid patients (HYPO) receiving increasing T_4 doses at 4-week intervals. Reference ranges are indicated by the *dashed lines*.

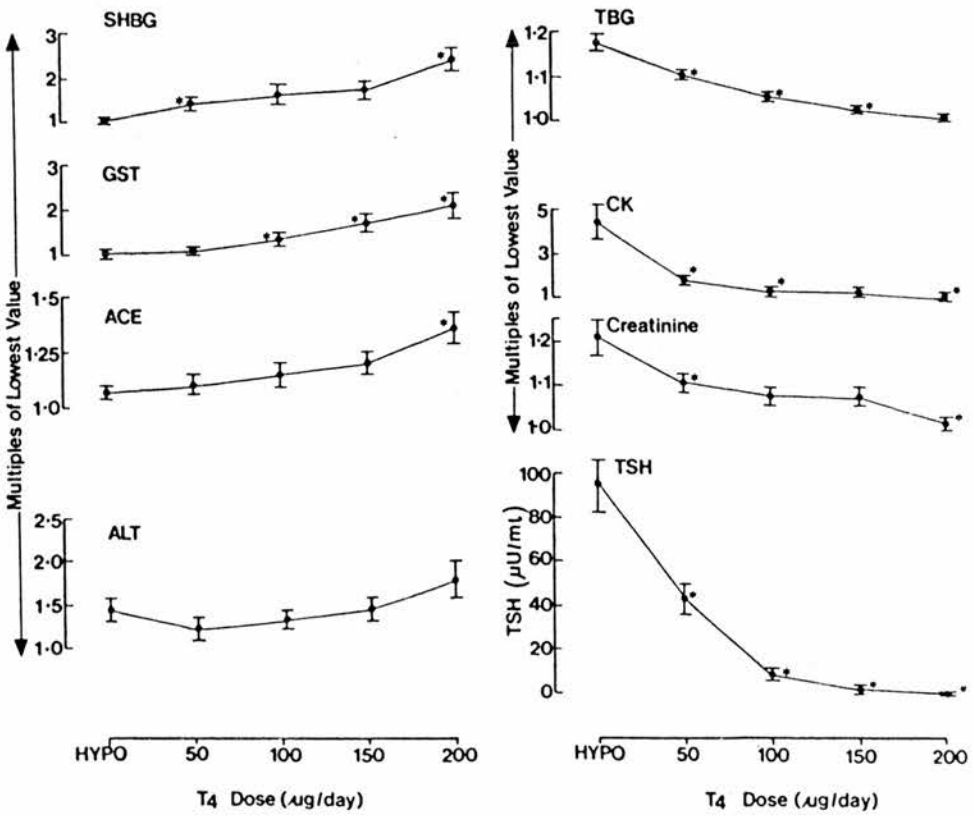

FIG. 2. Mean (±SE) peripheral tissue markers measured in serum from 21 hypothyroid patients (HYPO) receiving increasing T_4 doses at 4-week intervals. *, Significant difference from the previous dose ($P < 0.05$).

cial kits were used for SHBG (Farmos Diagnostica, SF-90460 Oulunsalo, Finland), TBG (RIA-gnost, Behringwerke AG, Marburg, Germany), and CK (Randox Laboratories Ltd., Crumlin, Co. Antrim, Northern Ireland) determinations. ACE was measured using a method based on that of Maguire and Price (23). The between-assay coefficients of variation for all of these analytes were less than 10%.

Laboratory-derived reference ranges were used for T_4 (5.6–12.0 μg/dl), T_3 (77–182 ng/dl), ALT (10–40 U/liter), GGT (5–35 U/liter), CK (30–150 U/liter), ACE (7–109 U/liter), creatinine (0.6–1.6 mg/dl), and GST (0.5–4.0 μg/liter). The manufacturers' suggested reference ranges were used for SHBG (30–90 nmol/liter) and TBG (10–42 mg/liter). Reference ranges for free thyroid hormones (free T_4, 0.8–1.8 ng/dl; free T_3, 0.28–0.55 ng/dl) and TSH IRMA (0.14–5.9 μU/ml) were derived from 97 clinically and biochemically euthyroid patients classified using total thyroid hormone levels and the TSH response to TRH, measured by RIA. Group I patient results were analyzed using the Wilcoxon matched pairs test, and group II results were analyzed using the Mann-Whitney test for unpaired data.

Results

Patients taking increasing doses of T_4 (group I)

Thyroid function tests. Serum total and free T_4 and T_3 concentrations were restored to normal at a lower T_4 dose than that necessary to suppress TSH to within the reference range (Fig. 1). Normal TSH (IRMA) results were achieved in 18 patients at the 50-, 100-, 150-, and 200-μg doses in 1, 8, 8, and 1 patients, respectively. Four patients could not tolerate the 200 μg/day dose for 4 weeks. Serum TSH (IRMA) became undetectable (<0.1 μU/ml) in 17 patients; this first occurred at the 100-, 150-, and 200-μg doses in 1, 7, and 9 patients, respectively. Serum free T_4 was above normal in all patients when TSH (IRMA) first became undetectable, whereas in most patients, serum T_4, T_3, and free T_3 levels were normal at that time. Conversely, if serum T_4, T_3, or free T_3 levels were raised, TSH (IRMA) was always undetectable, but 10 patients with increased free T_4 levels had normal TSH (IRMA) concentrations.

Serum markers from peripheral tissues. Dose-dependent increases in GST, SHBG, and ACE ($P < 0.05$) occurred (Fig. 2), and ALT levels at the 200 μg/day dose were higher than those at the 100 μg/day dose ($P < 0.05$). Significant reductions in TBG, CK, and creatinine also occurred with increasing T_4 dose (Fig. 2). GGT increased slightly but not significantly.

Levels of 1 or more of these markers became abnormal in a total of 15 patients. Abnormally high GST, SHBG, ALT, GGT, and ACE levels were found in 10, 7, 4, 3, and 3 patients, respectively. The occurrence of these abnormalities coincided with an undetectable TSH and raised free T_4 concentration in 9 patients, and in 4 further patients, TSH was undetectable and free T_4 was raised after the next incremental T_4 dose. By contrast, even at the highest dose, free T_3 and total T_4 and T_3

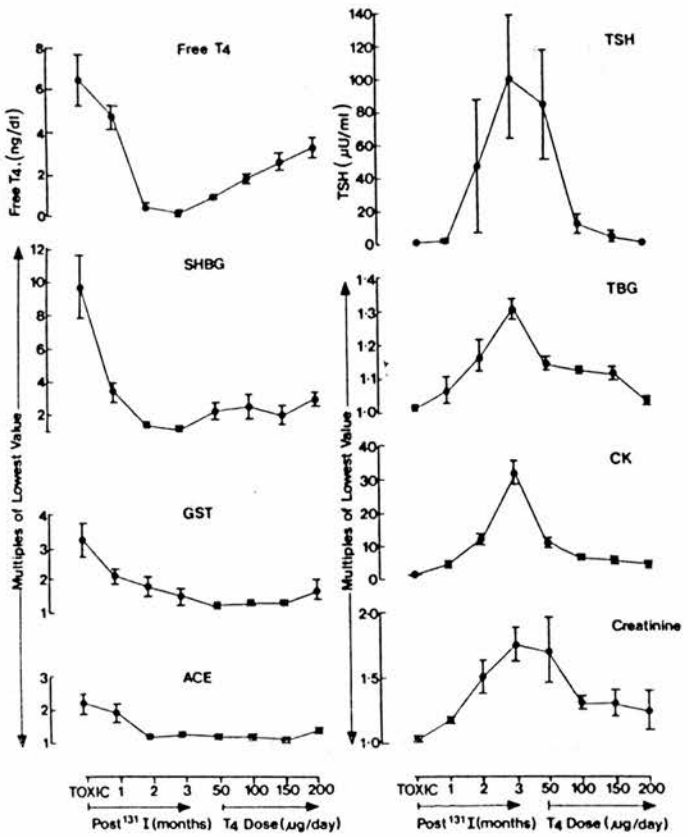

FIG. 3. Mean (±SE) peripheral tissue markers in five patients pre- and postradioiodine treatment for hyperthyroid Graves' disease and during T_4 therapy.

TABLE 1. Comparison of patient details and thyroid function tests in group II patients subdivided according to TSH concentrations

	Mean (SD or range)		
	Group A (low TSH)	Group B (normal TSH)	Group C (high TSH)
n	37	42	25
Dose (μg)	162 (60)[a]	127 (43)	116 (47)
Age (yr)	53 (13)	53 (14)	49 (14)
Wt (kg)	66 (14)	69 (13)	72 (12)
Duration (yr)	3.2 (0.3–15)	3.8 (0.3–22)	3.7 (0.5–21)
TSH (μU/ml)	<0.1[b]	1.4 (0.13–5.9)	24.3 (6.4–103)[b]
Free T_4 (ng/dl)	2.8 (1.4)[b]	1.7 (0.3)	1.2 (0.4)[b]
Free T_3 (ng/dl)	0.5 (0.2)[b]	0.4 (0.1)	0.3 (0.1)[c]
T_4 (μg/dl)	11.9 (3.5)[b]	9.5 (1.6)	7.4 (2.3)[b]
T_3 (ng/dl)	126 (28)[b]	105 (17.5)	96 (23.1)

Reference ranges: TSH, 0.14–5.9 μU/ml; free T_4, 0.8–1.8 ng/dl; free T_3, 0.28–0.55 ng/dl; T_4, 5.6–12.0 μg/dl; T_3, 77–182 ng/dl.

[a] $P < 0.005$ *vs.* group B.

[b] $P < 0.001$ *vs.* group B.

[c] $P < 0.05$ *vs.* group B.

were raised in only 8, 6, and 1 of these 15 patients, respectively.

In the nine patients who were studied after an additional 2 months, no further changes occurred in the measured thyroid and tissue parameters, except for a slight increase ($P < 0.05$) in SHBG.

The changes in tissue markers in five patients before radioiodine treatment and during T_4 replacement are shown in Fig. 3. The levels of these markers at the maximum T_4 dose were not as abnormal as when patients were overtly hyperthyroid. However, as with the complete group, there were trends toward higher SHBG, GST, and ACE and lower TBG, CK, and creatinine levels when these patients were taking 200 μg/day T_4 compared to the lower doses. Only one patient in this subgroup had levels outside reference limits.

Patients stabilized on a fixed dose of thyroxine (group II)

Thyroid function tests. Measurement of TSH (IRMA) divided the 104 patients receiving a constant T_4 dose into 3 groups: 37 patients with undetectable TSH levels (A), 42 with normal TSH levels (B), and 25 with raised levels (C; Table 1). Only 1 patient had a detectable TSH concentration (0.13 μU/ml) which was less than the absolute range (0.14–5.9 μU/ml) in a reference euthyroid population (12). This patient was included in group B. There was no bias toward a particular cause for the hypothyroidism between the groups or the duration of therapy, but patients in group A were receiving higher T_4 doses ($P < 0.005$) than those in group B. From Table 1, a mean reduction of 22% in T_4 dose would bring group A patients to the same mean dose as those in group B. Patients in group C had not been prescribed less T_4 than those in group B, but 7 were thought to be noncompliant with their treatment. The prescribed dose did not correlate significantly with the patient's age or weight. For detectable TSH values, Spearman's correlation coefficients ($P < 0.0001$) for TSH *vs.* free thyroid hormones were $r = -0.746$ for free T_4 and $r = -0.446$ for free T_3.

In group A, free T_4, T_4, free T_3, and T_3 were high in 32, 13, 12, and 1 patients, respectively. In group B, high free T_4 levels were found in 14 patients, and high T_4 levels were found in 3. One patient in group B had low T_4 and free T_3 results, and low levels of thyroid hormones were found in 7 group C patients.

Serum markers from peripheral tissues. The results for serum ALT, GST, GGT, CK, TBG, and SHBG are shown for the three groups in Fig. 4. For the TBG and SHBG data, all women taking an estrogen-containing oral contraceptive and men were excluded. Significantly higher ALT ($P < 0.001$), GST ($P < 0.02$), and GGT ($P < 0.05$) levels were found in group A than in group B. Lower CK levels were found in group A compared to group B ($P < 0.05$). Compared with group C, group A patients had higher SHBG ($P < 0.001$) and lower TBG levels ($P < 0.02$). ACE activities and creatinine levels did not differ significantly among the groups.

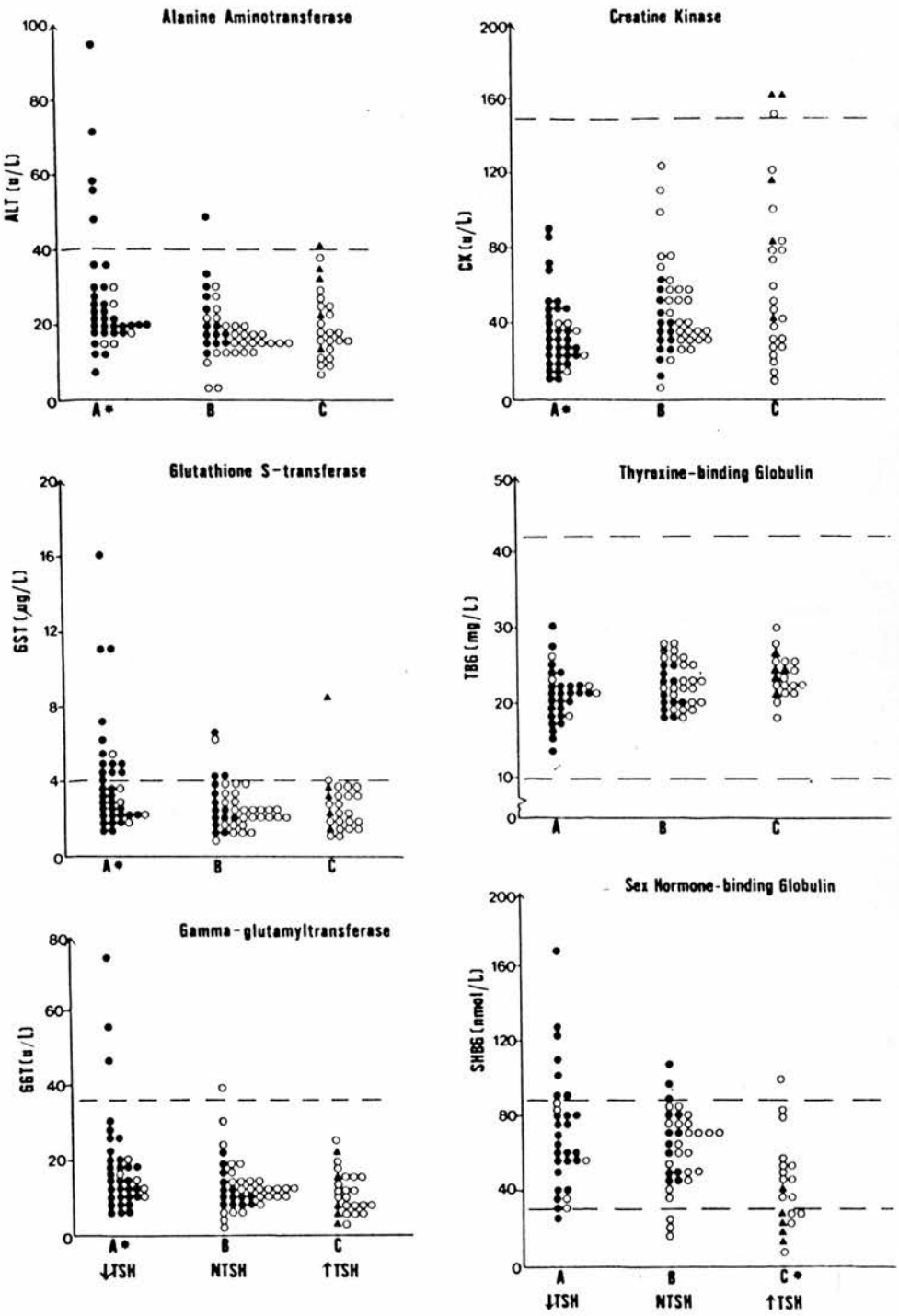

FIG. 4. Peripheral tissue markers in serum from 104 patients taking a fixed T_4 dose grouped according to TSH (IRMA) concentrations. Upper reference range values (ALT, GST, GGT, and CK) and reference ranges (TBG and SHBG) are indicated by the *dashed lines*. *, Significant difference from group B ($P < 0.05$). Values for patients with high (●), normal (○), and low (▲) free T_4 concentrations are indicated.

A total of 27 patients had an abnormally high level of at least 1 of the following: ALT, GST, GGT, and SHBG. Most of these patients had an undetectable TSH IRMA (67%) or raised free T_4 (85%), whereas free T_3 was raised in only 33%, and T_4 was raised in 26%.

Discussion

We clearly demonstrated a relationship between the serum markers used to assess tissue thyroid status and TSH levels measured by a sensitive assay in patients taking T_4. Such a relationship was not found between the serum markers and free T_3 measurements. Although the changes in SHBG, TBG, CK, and creatinine may reflect the return of peripheral tissues to normal from the hypothyroid state, some patients had abnormally high ALT, GST, GGT, SHBG, and ACE levels at the higher T_4 doses. These abnormalities were not as marked as those in patients with untreated overt hyperthyroid-

ism, but in addition to the complete suppression of TSH secretion, they provide evidence of a generalized tissue overexposure to thyroid hormones. The lack of complete concordance in the abnormalities we detected and pituitary suppression is not unexpected in view of the wide range of sensitivities and specificities to thyroid status of the tissue markers measured.

It might be argued that the changes observed in tissue markers in our group I patients were not related to the changes in T_4 dose, but, rather, to the duration of T_4 replacement. Indeed, although the majority of studies of effects of T_4 replacement were done using incremental doses at 3- to 6-week intervals (1–3, 6, 24), it has been reported that with increasing T_4 dosage a steady state may not be reached for several months (25). However, in nine patients in whom we monitored tissue markers and thyroid function tests for a further 2 months, we could not demonstrate any further changes, except a slight increase in SHBG levels with time. These criticisms of the steady state achieved in our group I patients cannot be related to the group II patients in whom constant T_4 doses had been taken for at least 3 months in all and for more than 6 months in the majority (>89%).

It has been suggested that sensitive TSH measurements cannot be used to indicate excessive treatment because of the increased sensitivity of the pituitary to T_4 compared to the sensitivities of other tissues (26). However, several reports have highlighted the need for maintaining pituitary responsiveness to TRH in T_4-treated patients, since mild hyperthyroidism cannot be detected by clinical means alone (1, 24, 27). Our findings indicate that TSH secretion can be used as a sensitive and representative test of peripheral tissue exposure to thyroid hormones in patients receiving T_4 replacement therapy. In group II patients, 35.5% would require, on the average, a reduction in dose of 22% to restore TSH levels to normal. These patients (group A) tended to have lower body weight than those with normal or high TSH levels, although the difference was not statistically significant. However, this observation in itself may reflect increased metabolism due to mild hyperthyroidism.

Many patients with normal serum total and free T_3 concentrations had evidence of tissue hyperthyroidism in this study. This is consistent with reports where other end organ responses were studied (7–11), but in disagreement with studies using clinical signs alone (5, 6). Although a greater proportion of patients with evidence of tissue hyperthyroidism had raised free T_4 concentrations than undetectable TSH (IRMA), a substantial proportion of patients required a high free T_4 level to reduce TSH secretion to normal. TSH secretion correlated more strongly with serum free T_4 than free T_3 concentrations, consistent with the known greater sensitivity of the pituitary to T_4 (14), but as shown in both groups, most patients with a normal serum TSH (IRMA) had normal total and free T_3 concentrations, indicating sufficient replacement for other tissues. A normal TSH (IRMA) concentration, therefore, indicates optimal T_4 replacement and makes measurement of serum thyroid hormones unnecessary. In patients in whom TSH (IRMA) is undetectable, we propose the use of a serum free T_4 measurement to provide a confirmatory test and an indication of the degree of overtreatment so that the T_4 dose could be adjusted accordingly.

There are some circumstances where the therapeutic aim in giving T_4 is to suppress TSH secretion completely, *e.g.* in patients with thyroid cancer or goiter due to Hashimoto's thyroiditis. However, in the majority, it would be advisable to achieve a normal TSH level, as measured by a sensitive assay. This would avoid the subclinical tissue changes that occur as a result of overexposure to T_4.

Acknowledgments

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Limitations of new thyroid function tests in pregnancy

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Summary

Sensitive immunoradiometric assays (IRMA) for TSH and radioimmunoassay (RIA) kits for free thyroid hormones (fT_4, fT_3) are becoming increasingly used for routine thyroid investigations. We have assessed these tests in 93 euthyroid pregnant women. Mean fT_4 and fT_3 values decreased with gestation by 24–27% and 14–35%, respectively, using several analogue RIA kits. Some patients had free hormone values which fell below the reference range derived from non-pregnant euthyroid patients. By contrast, the fT_4 concentrations measured by direct equilibrium dialysis fell by only 16% with all values within the reference range.

Serum non-esterified fatty acid (NEFA) levels (non-fasting) did not correlate with fT_4 and fT_3 but a spurious effect of serum albumin levels on the free hormone kits was suggested. TSH results showed that the majority of subjects had lower values measured by IRMA than by RIA. Three patients had basal TSH (IRMA) below the mean detection limit of the assay; this could have been falsely interpreted as indicating hyperthyroidism.

We conclude that, as with longer established thyroid function tests, special care must be taken in interpreting results of these new thyroid function tests in pregnancy.

Introduction

The diagnosis of hyperthyroidism in pregnancy may be difficult since the clinical features of tachycardia and heat intolerance may be due to the pregnancy itself. Measurement of serum total thyroxine (TT_4) and tri-iodothyronine (TT_3) may

provide little help in clarifying thyroid status, since high levels of circulating oestrogens induce the synthesis of thyroxine-binding globulin (TBG), thereby causing elevated TT_4 and TT_3 levels. Free thyroid hormone concentrations are independent of TBG levels, and have been advocated as alternative biochemical tests in this situation.

The TRH test discriminates hyperthyroid from euthyroid patients most reliably but it is time-consuming and may produce undesirable reactions in a small number of patients [1]. However, the recent introduction of the sensitive IRMA for measuring TSH has led to the suggestion that a basal TSH, by IRMA, may replace the TRH test [2].

Earlier studies of fT_4 in pregnancy using indirect dialysis methods have reported unchanged, increased and decreased values [3]. Recently, more reliable, direct dialysis methods have been developed in which the concentration of T_4 in the serum dialysate is measured directly by RIA. Using this technique, decreases in fT_4 with gestation have been shown by the majority of studies [4–6], although unchanged values were reported in one study of 39 pregnant women [7]. Direct dialysis remains the reference method for free hormone measurement.

Several commercial assays for fT_4 and fT_3 are now available; these are based on different principles and techniques. Many are RIA methods which use chemically modified radiolabels that do not bind appreciably to the endogenous serum binding proteins. Most studies using these analogue kit methods have shown decreased fT_4 and fT_3 levels as pregnancy progresses [7–10]. These findings have led to the use of trimester-related reference ranges which, in late pregnancy, overlap with the hypothyroid range. A TSH test is then required to confirm euthyroidism when low free thyroid hormone levels are found. Recent studies have shown that some analogue fT_4 assays are spuriously affected by changes in albumin and NEFA levels [10–13], but it is unclear whether the decreased albumin and raised NEFA levels reported in late pregnancy contribute to the low analogue fT_4 values.

In the present study, we have measured fT_4 levels in the three trimesters of pregnancy using a direct equilibrium dialysis RIA, and several commercial analogue methods for fT_4 and fT_3 analysis. The relationship between free hormone concentrations and albumin and NEFA levels has been investigated. We have also examined the possibility that basal TSH measurement performed by a highly sensitive IRMA could overcome the problems associated with total and free thyroid hormone measurements, and thus become the first-line test of thyroid function in pregnancy.

Patients and methods

Ninety-three pregnant women in the first (7–12 wk, $n = 37$), second (13–27 wk, $n = 32$) and third (28–40 wk, $n = 24$) trimesters of pregancy were studied. Concentrations of TT_4 and TT_3 in serum were measured by RIA [14]. fT_4 was measured by a direct dialysis method (fT_4(D)) as described by Giles [6] and by equilibrium RIA using kits supplied by Amersham International (Amerlex fT_4(A), Amerlex Magnetic fT_4(AM)), Corning Medical (Corning Magic fT_4(C)), Diagnostic Products Corp. (Coat A Count fT_4(CC)) and Becton Dickinson fT_4(BD); free T_3 was also

measured by kits supplied by Amersham International, Diagnostic Products Corp., and Becton Dickinson. TSH was measured in serum by a sensitive IRMA (Boots Cell-Tech), by a commercial RIA (Becton Dickinson), and by an in-house RIA [15].

Between-batch coefficients of variation (CV) over the concentration ranges measured were $< 10\%$ for all of the above assays except fT_4(D) which had a CV $< 15\%$. Reference ranges were derived for each method from 63 euthyroid, non-pregnant patients classified on the basis of clinical examination, normal TT_4, TT_3 and TRH tests.

Albumin was measured using a Bromocresol green dye-binding method on a RA1000 discrete analyser (Technicon Instruments Corp.) and NEFA levels using a Wako NEFAC kit (Wako Chemicals GmbH) on a Cobas Bio centrifugal analyser (Roche Diagnostics); the CV was $< 3\%$.

The effect of albumin on fT_4(D), fT_4(A) and fT_4(AM) levels was examined by adding increasing amounts of human serum albumin from Behring, Marburg, FRG

TABLE I

Thyroid function, albumin and NEFA tests in the three trimesters of pregnancy

	Trimester [a]						Mean % change
	I		II		III		I–III
TT_4(nmol/l)	136	(19.5)	152	(22.8) **	147	(24.9) *	+8%
TT_3(nmol/l)	2.2	(0.4)	2.6	(0.3) ***	2.5	(0.5) **	+15%
fT_4(pmol/l):							
Dialysis	13.4	(1.8)	12.1	(1.9) **	11.3	(1.6) ***	−16%
Amerlex	16.8	(1.6)	14.7	(3.1) ***	12.6	(2.2) ***	−25%
Amerlex Magnetic	16.1	(1.6)	14.3	(2.9) ***	11.8	(2.1) ***	−27%
Corning	23.2	(2.0)	20.2	(3.9) ***	17.6	(2.0) ***	−24%
Coat A Count	16.5	(2.3)	14.1	(3.4) ***	12.1	(2.1) ***	−27%
Becton Dickinson	12.8	(1.8)	11.5	(2.5) *	9.7	(1.6) ***	−24%
fT_3(pmol/l):							
Amerlex	6.2	(0.8)	5.0	(1.0) ***	4.0	(0.5) ***	−35%
Coat A Count	5.0	(1.0)	4.8	(0.9)	4.3	(0.8) **	−14%
Becton Dickinson	6.8	(1.2)	5.8	(1.0) ***	4.8	(0.9) ***	−29%
Albumin (g/l)	45	(2.4)	42	(3.3) ***	38	(1.7) ***	−16%
NEFA (mmol/l)	0.44	(0.26)	0.34	(0.17)	0.36	(0.11)	ns
TSH (mU/l):							
Boots Cell-tech IRMA	1.0	(0.8)	1.3	(1.0	1.6	(0.9) *	+65%
Becton Dickinson RIA	3.2	(0.9)	3.1	(1.1)	3.3	(1.0)	ns
In-house RIA	2.8	(0.7)	2.5	(0.6) *	2.6	(0.6)	ns

Free thyroid hormones were measured by equilibrium dialysis and a number of commercial kits as indicated. Serum TSH was measured by both RIA and IRMA methods.

[a] Mean values ($\pm$ SD) are shown for each trimester together with the mean percentage change in the analyte between trimester I and III. Significant differences in measurements made in the second and third trimester when compared with the first trimester are indicated by: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; ns, not significant.

of 100% electrophoretic purity (3–19 g/l) in 0.15 mmol/l NaCl to a low albumin plasma pool collected from hospital inpatients. Insufficient reagents were available to repeat the experiment with the other free hormone kits.

Student's *t* test was used for statistical analysis.

Results

Total thyroid hormone levels

TT_4 and TT_3 increased by 12% and 18% respectively between the first and second trimesters. These elevated levels were maintained in the third trimester (Table I).

Free thyroxine levels

Free T_4 levels fell significantly during pregnancy although the decrease between first and third trimesters was less by $fT_4(D)$ than by the kits (Table I). No $fT_4(D)$ values fell below the reference range. In some subjects, subnormal fT_4 concentrations

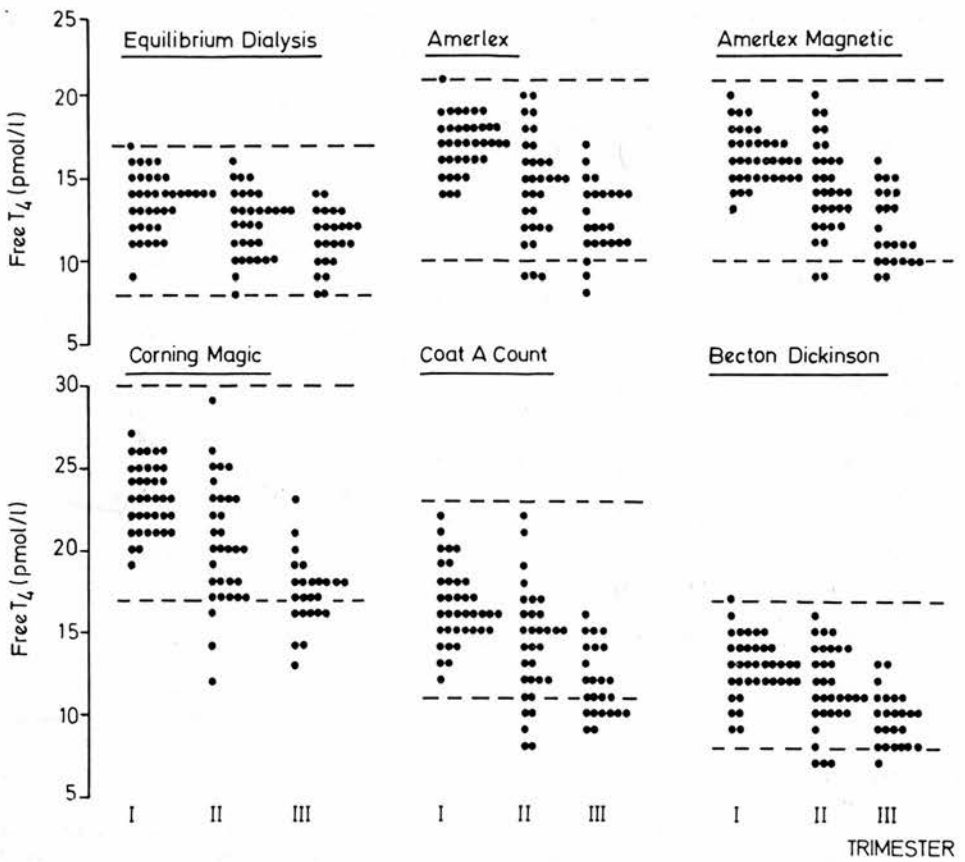

Fig. 1. fT_4 levels in the 3 trimesters of pregnancy. Reference limit (- - - - - -).

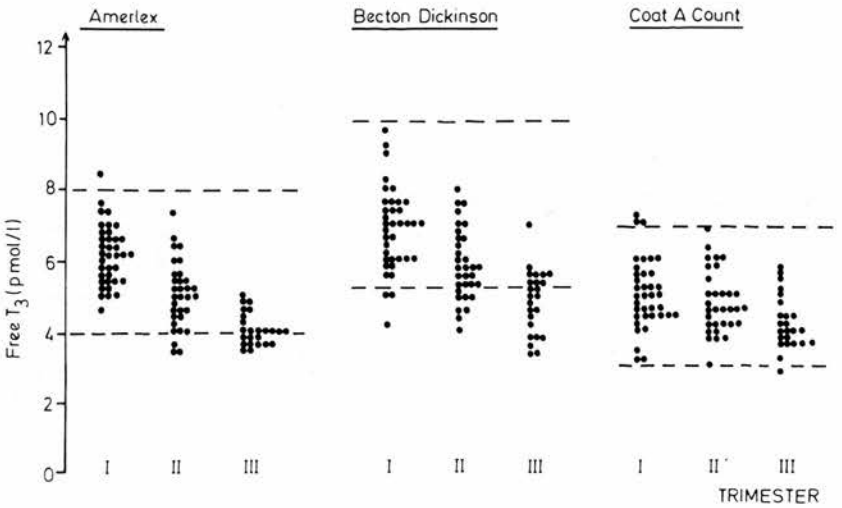

Fig. 2. fT_3 levels in the 3 trimesters of pregnancy. Reference limit (- - - - - -).

were found using the analogue kits although the percentage of these subnormal levels varied with the kit used; i.e. fT_4(A) 5%, fT_4(AM) 4%, fT_4(C) 12%, fT_4(CC) 14%, fT_4(BD) 4% (Fig. 1).

Free tri-iodothyronine levels

Mean fT_3 levels also decreased during pregnancy. There were marked differences in the magnitude of this fall measured by the various kits, with fT_3(CC) showing the smallest change. The percentage of values falling below the reference range were: fT_3(A) 14%, fT_3(BD) 35%, fT_3(CC) 1% (Fig. 2).

TABLE II

The effect of added albumin on free T_4 measurement

Albumin (g/l)	fT_4(A)	fT_4(AM)	fT_4(D) (pmol/l)
31 *	8.6	8.4	9.4
34	9.1	9.2	9.2
37	10.3	9.7	8.6
40	11.3	10.4	8.9
44	12.1	11.9	8.6
50	13.9	12.9	9.0
ΔfT_4/(7 g/l albumin)	+1.9	+1.7	ns

Free T_4 levels measured by two analogue RIA methods and by equilibrium dialysis in a low albumin plasma pool before, *, and after albumin addition to give the final concentrations shown. ΔfT_4 represents the calculated effect on fT_4(A) and fT_4(AM) values when a change of 7 g/l in albumin concentration occurs, as seen in pregnancy.

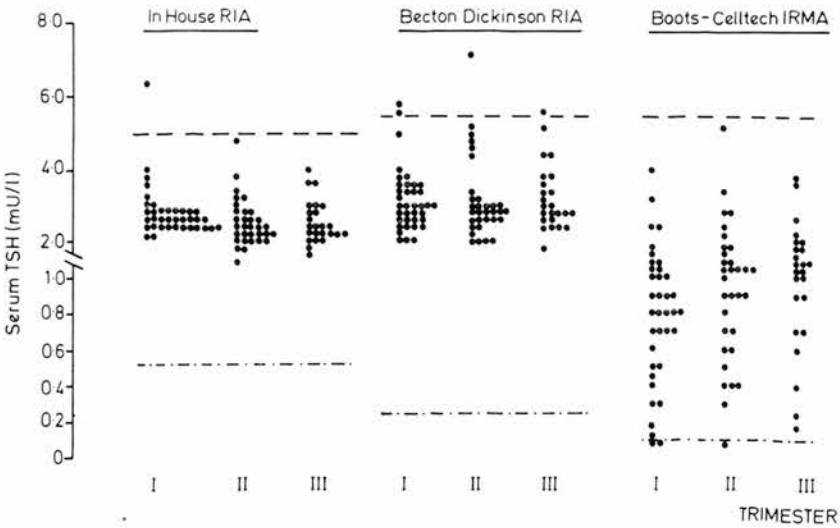

Fig. 3. TSH levels in the 3 trimesters of pregnancy. Upper reference limit (- - - - -). Mean detection limit (– · – · –).

Albumin and NEFA levels

Mean albumin levels decreased by 16% during pregnancy but NEFA (non-fasting) levels did not change significantly (Table I). Free T_4 correlated strongly ($p < 0.001$) with albumin levels in the second trimester, as measured by all the kits, whereas fT_4(D) did not. Free T_3(A) and fT_3(BD) also showed this correlation ($p < 0.001$, $p < 0.01$) unlike fT_3(CC). None of the free hormone tests correlated with the NEFA levels found in these non-fasting subjects.

The in vitro effect of added albumin on free hormone levels

The addition of albumin to a low albumin plasma pool did not significantly affect fT_4(D) values. However, significant increases were found with the analogue kits tested (Table II). The mean decrease in albumin of 7 g/l during pregnancy could therefore cause an additional decrease of 11% in fT_4(A) and fT_4(AM) values.

TSH levels

The TSH results for each trimester are shown in Fig. 3. The detection limit shown for each of the assays is the mean from 5 consecutive assays and defined as the minimum concentration distinguishable from zero at the 95% confidence level. By this criterion, the TSH IRMA was ten times more sensitive than the in-house RIA. The TSH IRMA results were lower than results by RIA in all trimesters, and 3 values fell below the mean detection limit. There was a significant increase in mean TSH IRMA between the first and the third trimesters, but TSH(BD) showed no change and the TSH (in-house) values significantly decreased between the first and the second trimesters (Table I).

Discussion

The decrease in free thyroid hormones with gestation shown by the analogue kits used in this study is in general agreement with the manufacturers' data and other studies. However, the magnitude of the fall and proportion of values below non-pregnant reference limits described here differs from some previous reports. For example, Tuttlebee found 55% of third trimester women to have fT_4(A) below the reference range compared to only 8% in our study [8]. Our reference dialysis method confirmed that a decrease in fT_4 does occur throughout pregnancy, but the mean fall of 16% was considerably less than that shown by any of the kit fT_4 values (24–27%); no fT_4(D) values fell below the reference range. The decrease in fT_3 measured by analogue kit methods was more diverse (14–35%), and was probably due to greater differences in the binding characteristics of the different T_3 radiolabelled analogues.

Significant rises in NEFA levels have been reported in fasting, pregnant women [16] and it has been suggested that low fT_4 values, obtained by analogue kit methods, might result from the displacement of residually-bound labelled T_4 analogue from albumin by NEFA [11]. However, Wilkins and Midgley estimated that even the highest levels seen in the third trimester would only reduce fT_4(A) by 4% at most [17]. The majority of studies, as here, have not used fasting subjects; under nonfasting conditions, we could not demonstrate a change in NEFA levels with gestation, nor any correlation between NEFA and fT_4 levels. Since any effect of NEFA on the fT_4 equilibrium should be dependent on the NEFA concentration at the time of sampling, and not on the fasting NEFA level, our data indicate that changes in NEFA concentrations are not responsible for the subnormal fT_4 values shown by the analogue kits in pregnancy.

We have clearly demonstrated, by equilibrium dialysis, that fT_4 concentrations do fall during pregnancy, so comparison of fT_4 with any other plasma constituent which also falls during pregnancy is likely to produce a correlation. A strong correlation between albumin and fT_4 concentration was observed in the second trimester using the analogue kits, but not with equilibrium dialysis. However, this probably reflects the poorer precision of the fT_4(D) method and cannot be interpreted as an albumin effect on the kit assays. Nevertheless, the difference in the magnitude of the fall in fT_4 during pregnancy, as measured by these two techniques, does suggest that some factor may be causing an additional fall in fT_4, when measured by the analogue kit assays.

Albumin has been implicated as this factor by other studies where the addition of albumin to analbuminaemic sera increased fT_4(A) but not fT_4 dialysis results [12]. We have shown that increasing the albumin concentration of a low albumin pool by small increments, to give values within the normal range (36–47 g/l), did not affect fT_4(D) values but increased the fT_4 values measured by the analogue kit methods tested. The fT_4(AM) kit, unlike the fT_4(A) kit, contains an 'albumin blocker' which, in a study by the manufacturer, reduced the fall in fT_4 seen in pregnancy from 30% (fT_4(A)) to 15% (fT_4(AM)) (Amersham Technical Bulletin). We did not observe this difference, and both Amersham kits were affected by added albumin in a parallel manner in this study. Correction of our pregnancy data for this albumin effect

would reduce the fall seen in fT_4 during pregnancy to 14% (fT_4(A)) and 16% (fT_4(AM)), resulting in closer agreement with the dialysis method. Our data therefore suggest that low albumin levels in late pregnancy may exaggerate the physiological fall in free hormones as measured by analogue kits.

Abdalla et al. showed a decrease in fT_4(A) in postmenopausal women treated with oestrogens, suggesting that oestrogens may be the physiological cause of the low free T_4 values in pregnancy [18]. An increase in the maximal nuclear binding capacity for T_4 and T_3 in mononuclear blood cells has been demonstrated in late pregnancy, together with reduced fT_4(A) and fT_3(A), suggesting a mechanism by which euthyroidism is maintained in these subjects at the tissue level [19].

It has been suggested that sensitive basal TSH IRMA tests could replace the TRH test and serve as the first-line test of thyroid function [2]. Our TSH IRMA results, however, were considerably lower in pregnant subjects than those measured by two RIA methods reported to have negligible cross-reaction to HCG. The fact that no TSH results by RIA were undetectable suggests that a specificity problem with pregnancy sera was still present in these assays. Of particular concern was the observation that 3 of our patients had undetectable levels of TSH when measured by IRMA. These results may have been incorrectly interpreted as indicating hyperthyroidism. High HCG levels in early pregnancy are known to cause a reduction in TSH IRMA values due to cross-reactivity with the solid-phased monoclonal antibody. This is claimed by the manufacturer to result in a mean 13% decrease in TSH levels. Our data suggest greater interference, since mean levels between the first and third trimesters differed by 35%.

What can we conclude concerning the value of these new tests when assessing thyroid status in pregnancy? Firstly, it would appear that, irrespective of the particular analogue kit method used, free hormone levels below the non-pregnant reference range will be obtained in pregnancy. Interpretative difficulties will therefore occur if such methods are used without trimester-related reference ranges. In some cases, a TSH test may be necessary to distinguish a pathological from a methodological cause of low free thyroid hormones. Secondly, the sensitive TSH IRMA described here also has limitations in some patients, particularly in early pregnancy, since an undetectable TSH IRMA with a normal free thyroid hormone level may be due either to subclinical hyperthyroidism or to HCG interference in the TSH assay in a euthyroid patient. The possibility that low albumin levels in a hyperthyroid pregnant woman could produce normal free thyroid hormone results by RIA should also be considered. Although a normal TSH IRMA result in a pregnant patient is good evidence against hyperthyroidism, an undetectable level requires further investigation, by means of the TRH test.

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An improved approach to thyroid function testing in patients with non-thyroidal illness

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Summary

We have compared the results of serum thyrotrophin (TSH) measurements using a sensitive immunoradiometric assay (IRMA) with those of conventional thyroid function tests in 299 hospital inpatients with a range of non-thyroidal illnesses. Levels of total thyroxine (T_4), free T_4, total tri-iodothyronine (T_3) and free T_3 in the hypothyroid range were recorded in 8%, 15%, 19% and 49% of patients, respectively, whereas TSH (IRMA) was abnormally low in 1%. Furthermore, basal TSH (IRMA) accurately predicted the result of the thyrotrophin-releasing hormone test in 72 of the 74 patients in whom this test was performed and, unlike thyroid hormone measurement, identified patients with subclinical thyroid disease.

It would appear that a single basal TSH (IRMA) measurement is the most appropriate screening test for thyroid dysfunction in patients with concomitant acute or chronic illness.

Introduction

Measurements of both total and free thyroid hormones frequently produce misleading results in patients with acute or chronic non-thyroidal illness particularly if used as screening tests for thyroid disease [1]. Systemic illness affects thyroid hormone measurement due to alterations in binding protein concentrations and affinities, the increased peripheral conversion of T_4 to T_3 and to drug effects [2]. Changes in albumin concentration can have marked effects on one-step analogue assays for free thyroid hormones and the validity of these measurements is now seriously questioned [3]. The poor diagnostic accuracy of thyroid hormone measure-

ments in such patients may lead to unnecessary further investigations and, occasionally, to inappropriate thyroxine replacement therapy or anti-thyroid treatment [4]. Serum thyrotrophin (TSH) levels measured by radioimmunoassay, and the TSH response to thyrotrophin-releasing hormone (TRH), are more reliable indicators of thyroid status in these patients [1]. The TSH response to TRH, although modified by age [5], illness such as chronic renal failure [6] and drugs such as corticosteroids, rarely gives false positive results. However, it has not been widely accepted as a screening test due to the need for an intravenous injection and for a second sample of blood to be taken 20 min later.

We have shown that basal serum TSH, measured by a sensitive immunoradiometric assay (IRMA), can accurately predict the TSH response to TRH when TSH is measured by radioimmunoassay [7] and have suggested that basal serum TSH (IRMA) measurement could be used as a screening test in patients with suspected thyroid disease [8]. The patients in these previous reports had all been referred to an out-patient clinic with suspected thyroid disease alone. The purpose of the present study was to assess whether the same approach could be applied to hospital in-patients with non-thyroidal illness.

Patients and methods

A total of 299 patients were studied; none had a past history or clear clinical evidence of thyroid disease. Group I patients comprised 264 consecutive admissions to a general medical ward (137 males, 127 females), who were receiving a wide range of drug treatments. Cardio/cerebrovascular, respiratory, liver and gastrointestinal problems were the reason for admission in 40%, 19%, 9% and 8%, respectively. The remaining 24% of patients were admitted with a variety of other illnesses. The ages of these patients showed a modal distribution (range 16–96 yr, mode 75 yr). Group II consisted of 35 patients (15 males, 20 females) with chronic renal failure who were receiving treatment by intermittent haemodialysis; they had an age range of 14–70 yr (mean 45 yr).

Serum total thyroxine (T_4) and triiodothyronine (T_3) were measured in all patients by radioimmunoassay (RIA) [9]. Free T_4 (fT_4) was measured by the Amerlex kit (Amersham International, UK) and free T_3 (fT_3) by the Becton Dickinson kit (Becton Dickinson UK Ltd). Basal serum TSH was measured by a sensitive IRMA (Boots-Celltech, UK) and had a minimum detection limit of 0.1 mU/l. In addition, the TSH response 20 min after the intravenous administration of 200 μg TRH was measured in 74 of the patients in Group I using a TSH RIA which had a minimum detection limit of 0.9 mU/l [10]. The upper limit of normal was 5.7 mU/l and the normal range of response 20 min after TRH was 3.9–25.3 mU/l for the TSH RIA [11]. A TSH increment of < 1 mU/l above basal was considered to be an absent response. Between-assay coefficients of variation (CV) were $< 8\%$ for all of these methods.

Reference ranges were derived from 91 female and 6 male patients (age range 16–77 yr, mean 43), referred to an endocrine clinic with suspected thyroid disease but in whom no clinical evidence of thyroid disease was present when examined by

one experienced Endocrinologist. In addition, basal serum TSH levels and the TSH response to TRH measured by RIA, was normal in every case. Reference ranges were: 60–145 nmol/l for T_4, 1.0–2.7 nmol/l for T_3, 10.0–22.5 pmol/l for fT_4 and 5.3–10.0 pmol/l for fT_3. Basal serum TSH concentrations by IRMA ranged from 0.14–5.9 mU/l.

The Mann–Whitney test for unpaired data was used for statistical analysis.

Results

Abnormalities in thyroid function tests in patients with nonthyroidal illness

Figures 1 and 2 show the results of serum T_4, fT_4 and basal TSH IRMA tests in Group I and Group II patients, respectively. In Group I, a substantial number of patients had low T_4 and fT_4 levels but only four had unequivocably high TSH levels and on recall showed clinical evidence of hypothyroidism; these patients are now receiving treatment with thyroxine. One other patient, who died subsequently, had low thyroid hormone results and a TSH IRMA level of 7.4 mU/l. A further 10 patients (8 female, 2 males; aged 56–86 yr) had basal TSH levels > 7.0 mU/l but normal T_4 levels; fT_4 levels were low in 5 of these patients.

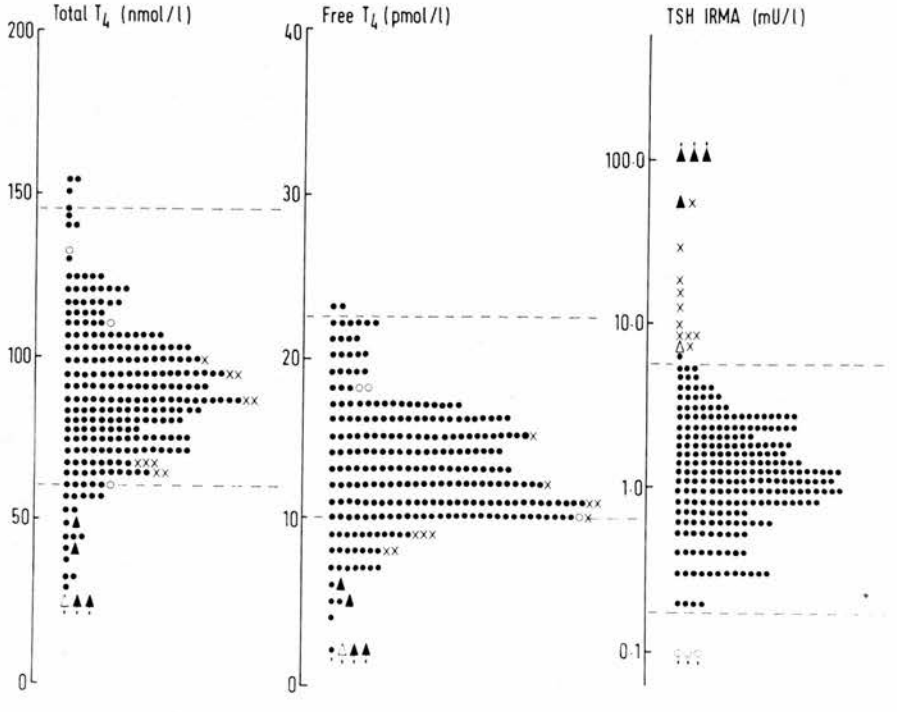

Fig. 1. Serum T_4, fT_4 and basal TSH IRMA results in patients from Group I. Results for patients with undetectable TSH IRMA (○), overt hypothyroidism (▲) and elevated TSH IRMA but normal T_4 (×), are as indicated. One patient (△) had septicaemia and later died.

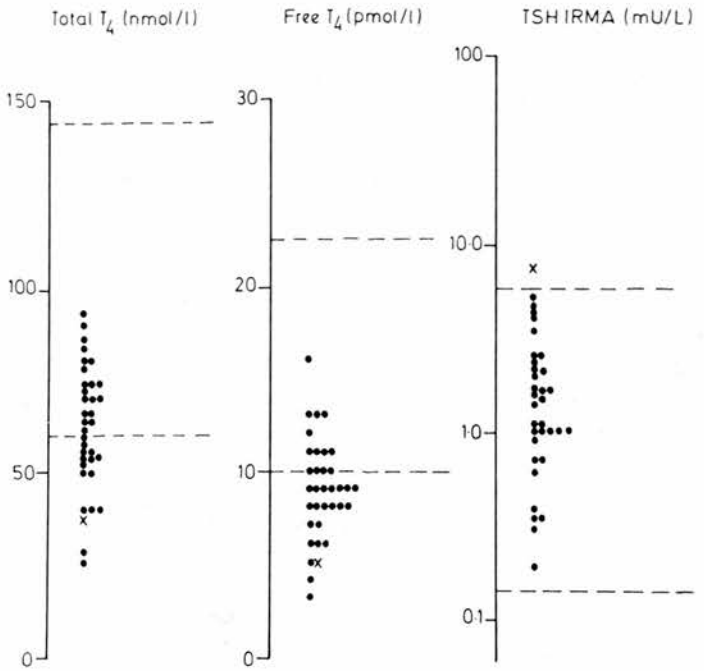

Fig. 2. Serum T_4, fT_4 and basal TSH IRMA in patients with chronic renal failure (Group II). The results for a patient with an elevated TSH IRMA (×) are as indicated.

In those patients with low T_4 but normal TSH concentrations, the main diagnoses were; liver dysfunction ($n = 3$), respiratory disease ($n = 3$), cardiovascular disease ($n = 3$), diabetes ($n = 2$), epilepsy ($n = 2$), acute renal failure ($n = 1$), nephrotic syndrome ($n = 1$) and ulcerative colitis ($n = 1$). Four patients were drug free, the two epileptic patients were taking phenytoin or sodium valproate and the remainder were receiving digoxin, diuretics or antibiotics. Serum total protein concentrations less than 60 g/l and albumin concentrations less than 36 g/l were found in 5 and 9 patients, respectively.

Two women in Group I, who had elevated T_4 and normal fT_4 levels, were taking an oestrogen-containing oral contraceptive.

Three patients had undetectable basal TSH levels by IRMA; the results of their other tests are shown in Table I (patients 1–3). One patient had borderline high T_4 and fT_4 levels on later testing by her general practitioner, consistent with subclinical hyperthyroidism; the remaining two patients were lost to follow-up.

In the group II patients, the prevalence of abnormal T_4 and fT_4 results was even greater, although only one patient had an abnormal TSH IRMA (Fig. 2).

Table II compares the number of abnormal results by each test in the two patient groups. The TSH IRMA clearly showed the lowest number of abnormal results.

A significant decrease in basal TSH IRMA was shown in Group I patients ($p = 0.02$) but not in Group II patients when compared to the reference group after

TABLE I

Patients with undetectable basal TSH (IRMA) or an absent TSH (RIA) response to TRH

Patient [a]	Sex	Age	Diagnosis	T_4 nmol/l (60–145)	fT_4 pmol/l (10–22.5)	T_3 nmol/l (1.0–2.7)	fT_3 pmol/l (5.3–10.0)	TSH IRMA mU/l (0.14–5.9)
1 [b]	F	69	Stroke	132	18.0	2.1	9.5	< 0.1
2	F	82	Wedge Fracture	110	18.0	1.8	7.9	< 0.1
3	M	33	Alcohol withdrawal fit	60	9.8	1.0	5.1	< 0.1
4	F	75	Chest Infection	102	17.0	0.8	2.2	0.5
5	F	67	Chronic Bronchitis	116	22.0	1.2	4.3	0.3

[a] Patients 1–3 did not have TRH tests performed; patients 4 and 5 had absent TSH responses to TRH but later died.
[b] Borderline-high thyroid hormone results on subsequent testing.

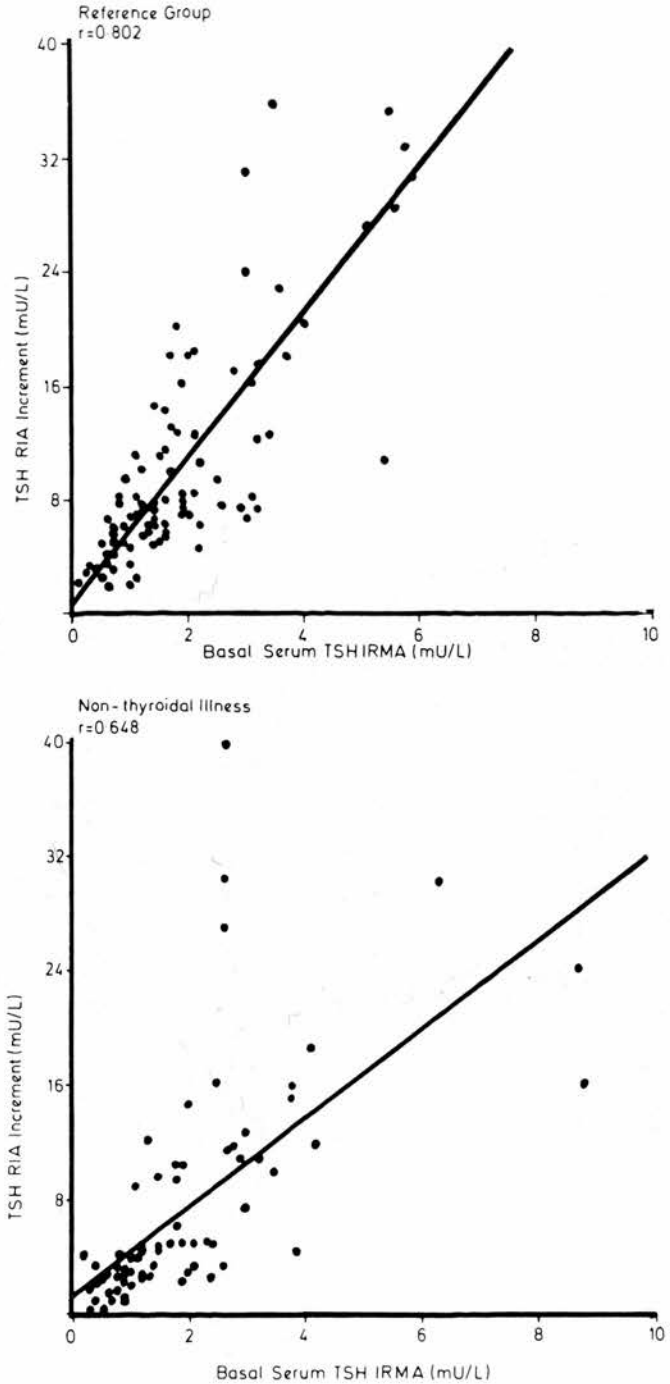

Fig. 3. The correlation of the serum TSH RIA increment after TRH with basal serum TSH IRMA in the reference group and in patients with nonthyroidal illness (Group I).

TABLE II

The number of patients with abnormal thyroid funciton tests

Result	Group I (264 patients)		Group II (35 patients)	
	Low	High	Low	High
T_4	21 (8%)	3 (0.1%)	15 (43%)	0 –
fT_4	40 (15%)	2 (< 0.1%)	24 (69%)	0 –
T_3	50 (19%)	0 –	10 (29%)	0 –
fT_3	128 (49%)	2 (< 0.1%)	25 (71%)	0 –
TSH IRMA	3 (1%) [a]	15 (6%)	0 –	1 (3%)

[a] Undetectable.

excluding the abnormal TSH results discussed above. Further analysis showed that this decrease was confined to males ($p < 0.002$) and was age-related ($r = -0.265$, $p < 0.01$).

Relationship between basal TSH (IRMA) and TSH response to TRH in nonthyroidal illness

There was a highly significant correlation between the basal TSH (IRMA) and the TSH (RIA) increment after TRH in the reference group ($r = 0.802$; $p < 0.001$) and in 74 Group I patients ($r = 0.648$, $p < 0.001$) although the scatter of points was greater in the latter (Fig. 3). Diminished TSH (RIA) responses to TRH of < 3.9 mU/l at 20 min, were found chiefly in the older male patients (Mann–Whitney, $p < 0.05$). In those patients with diminished responses ($n = 30$), T_4 and fT_4 concentrations were low in 10% and 13%, respectively.

Two patients had absent TSH (RIA) responses to TRH although basal TSH was detectable by IRMA (Table I, patients 4, 5). Both were women who later died in hospital.

Discussion

Thyroid function tests are frequently requested in hospital in-patients often with little clinical justification and despite reports that the diagnostic yield of this 'screening' approach only proves rewarding in the elderly, where there is an increased prevalence of thyroid disease [12–14]. However, since such requests are likely to continue, there is a need for a screening test which is unaffected by the presence of non-thyroidal illness.

In this study the basal serum TSH measurement by IRMA gave the lowest number of abnormal results out of the tests performed in the group I patients, most of whom were elderly. This was even more marked in those patients with chronic renal failure (group II), where reliable tests of thyroid status are essential since clinical features of hypothyroidism may be present. The possibility that patients with thyroid disease were missed using this test has to be considered. However, the number of abnormal results using basal TSH (IRMA) agrees well with the reported prevalence of 5% for thyroid disease in the elderly [15].

It has been argued that TSH may be inappropriately low in glucocorticoid treated patients [16], during phenytoin therapy [17] and in fasting subjects [18]. In this study, only one patient with a low T_4 and normal TSH received phenytoin and none were receiving glucocorticoids. It has also been shown that the suppressive effect of fasting on TSH secretion is only sufficient to normalise TSH secretion in mild rather than overt hypothyroidism [18]. Although we cannot exclude such a suppression in our low T_4 patients, the basal TSH IRMA levels in those patients with low T_4 but normal TSH, were not significantly higher than the reference group. The low T_4 or fT_4 levels in the presence of a normal TSH IRMA could equally be due to the low serum protein and albumin concentrations present and the drug therapy used in these patients.

Four patients with overt hypothyroidism were identified by all the tests. A further 10 group I patients, 8 of whom were elderly females, had > 7.0 mU/l basal TSH but normal T_4 and T_3 levels, consistent with the presence of subclinical hypothyroidism. Elevations in basal TSH levels have been reported during the recovery phase after systemic illness [19]. This is unlikely to account for the raised results found here, as all blood samples were taken on admission to hospital. In addition, 5 of the 10 patients had low fT_4 levels and these could not be attributed to low serum albumin or medication known to lower Amerlex fT_4 values. It would be expedient, however, to repeat the TSH measurement in such patients after resolution of the systemic illness before T_4 replacement therapy were considered.

An illness-associated reduction in basal TSH levels has been reported in cancer patients undergoing bone marrow transplantation [20], but in general this reduction was not observed in either of our patient groups. In addition, low T_4 or fT_4 levels did not occur more frequently in those Group I patients with diminished TRH responsiveness. Transient secondary hypothyroidism may be a feature of more critically ill patients, for example, in an intensive care unit, where routine investigation of thyroid status would not be a major concern. In chronic renal failure, pituitary TSH secretion is inappropriately low for the measured thyroid hormone levels. Whether this is due to the presence of altered binding proteins and endogenous inhibitors which affect conventional measurement of T_4 and fT_4 [2] or to non-pituitary tissues being maintained in a state of hypothyroidism as a physiological response to the illness [6], remains unclear.

Our study revealed 1% of patients with undetectable serum TSH (IRMA). One patient had continued biochemical evidence of hyperthyroidism after discharge. The other two patients were lost to follow-up and we were unable to determine if their undetectable TSH (IRMA) was due to subclinical hyperthyroidism or to their non-thyroidal illness. Nevertheless, in the population studied, fewer patients required confirmatory tests of thyroid function using TSH (IRMA) as the first-line test, than if T_4 or fT_4 levels had been used. Also, T_4 measurement does not detect patients with subclinical thyroid disease who may benefit from follow-up and later treatment [21–23]. A measurement of basal TSH by IRMA has the advantage of identifying such patients [8].

We have shown that the strong positive correlation between basal TSH (IRMA) and the TSH response to TRH is generally maintained in patients with non-thyroidal

illness, providing a sound theoretical basis for the use of basal TSH (IRMA) in the investigation of these patients. Disparity occurred in only 2 out of 74 patients tested where the basal TSH (IRMA) was detectable but the TSH (RIA) response to TRH was absent. As both patients subsequently died, we could not repeat the tests on recovery from the systemic illness. However, in a recent study, Davies et al showed that 80% of elderly, euthyroid patients with non-thyroidal illness who had no TSH response to TRH on presentation, showed TSH responsiveness at subsequent follow-up [24]. The majority of their patients (9/10) had detectable basal TSH levels. The use of a basal TSH IRMA measurement may, therefore, provide a more specific test to exclude thyroid disease than the TRH test in patients with non-thyroidal illness.

The measurement of basal serum TSH by a sensitive assay should supercede conventional thyroid function tests as the first-line test of thyroid function in patients admitted to a general medical ward.

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